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     FILE 'CA, BIOSIS, MEDLINE' ENTERED AT 15:05:27 ON 21 JUN 2002
L1          5747 S TISSUE ADHESIVE?
L2         63171 S FIBRONECTIN?
L3            98 S L1 AND L2
L4             0 S L3 AND (ELASTASE INHIBITOR? OR EGLIN?)
L5            48 S L2 AND (ELASTASE INHIBITOR? OR EGLIN?)
L6            39 S L2 (P) (ELASTASE INHIBITOR? OR EGLIN?)
L7            17 DUP REM L6 (22 DUPLICATES REMOVED)
L8            20 S FIBINOGEN?
L9         88900 S FIBRINOGEN?
L10          146 S L1 (P) L9
L11            1 S (ELASTASE INHIBITOR? OR EGLIN?) AND L10
L12            1 S L1 AND L9 AND (ELASTASE INHIBITOR? OR EGLIN?)
L13           42 S FIBRINOGEN? AND (ELASTASE INHIBITOR? OR EGLIN?)
L14           26 S FIBRINOGEN? (P) (ELASTASE INHIBITOR? OR EGLIN?)
L15           15 DUP REM L14 (11 DUPLICATES REMOVED)
L16            1 S L1 AND (ELASTASE INHIBITOR5 OR EGLIN)
L17      2091458 S SURGERY OR WOULD OR (BLOOD CLOTTING)
L18         4007 S ELASTASE INHIBITOR? OR EGLIN
L19          129 S L18 AND L17
L20           79 S L18 (P) L17
L21            1 S L1 AND L18
L22        88903 S L20 AND PLASMINOGEN? OR FIBRINOGEN?
L23            1 S L20 AND FIBRINOGEN?
L24            3 S L20 AND PLASMINOGEN?

=> s surger? or wound? or (blood clotting?)
L25     1699572 SURGER? OR WOUND? OR (BLOOD CLOTTING?)

=> s l25 and l18
L26           61 L25 AND L18

=> s l25 (p) l18
L27           31 L25 (P) L18
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Freeform Search

Database: US Patents Full-Text Database; US Pre-Grant Publication Full-Text Database; JPO Abstracts Database; EPO Abstracts Database; Derwent World Patents Index; IBM Technical Disclosure Bulletins

Term: 113 same (elastase inhibitor$5 or eglin)

Display: 10 **Documents in Display Format:** - **Starting with Number** 20

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Main Menu | Show S Numbers | Edit S Numbers | Preferences | Cases

Search History

DATE: Friday, June 21, 2002 Printable Copy Create Case

Set Name side by side	Query	Hit Count	Set Name result set
DB=USPT,PGPB,JPAB,EPAB,DWPI,TDBD; PLUR=YES; OP=ADJ			
L15	l13 same (elastase inhibitor$5 or eglin)	56	L15
L14	l13 and (elastase inhibitor$5 or eglin)	292	L14
L13	SURGERY OR WOUND OR (BLOOD CLOTTING)	522990	L13
L12	SURGERY OR WOULD OR (BLOOD CLOTTING)	83802	L12
L11	l4 and (elastase inhibitor$5 or eglin)	3	L11
L10	l9 and (elastase inhibitor$5 or eglin)	0	L10
L9	fibrin adhesive	166	L9
L8	l5 and (elastase inhibitor$5 or eglin)	3	L8
L7	l6 and ((elastase inhibitor$5) or eglin)	1	L7
L6	l5 and plasminogen	39	L6
L5	l4 same fibrinogen	198	L5
L4	tissue adhesive	1097	L4
L3	fibrinogen same elastase inhibitor	10	L3
L2	fibrinogen same eglin	2	L2
L1	fibrinogen and eglin and plasminogen	13	L1

END OF SEARCH HISTORY

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L6: Entry 18 of 39 File: USPT Feb 25, 1997

DOCUMENT-IDENTIFIER: US 5605887 A
TITLE: Therapeutic fibrinogen compositions

Abstract Paragraph Left (1):
A therapeutic composition effective on contact with thrombin at a site of treatment in a patient as a tissue adhesive, hemostat or sealant, said composition comprising non-autologous, non-single donor mammalian fibrinogen that is capable of polymerizing when provided in solution at said site at a concentration of about 30 mg/ml thereof or less, to a fibrin network having therapeutically effective strength, wherein said composition contains less than about 30% (w/w), based on total protein mass present therein, of proteins other than fibrinogen, and further comprises a sufficient amount of one or more low molecular weight physiologically-compatible solutes such that said composition, if formulated as a lyophilized material, can be reconstituted therefrom at room temperature in sterile water for injection in about 30 minutes or less, at about 25 mg/ml of said fibrinogen. Additionally, methods for producing and maintaining said composition, and methods for the use thereof.

Brief Summary Paragraph Right (1):
This invention relates to therapeutic compositions comprising non-autologous non-single donor fibrinogen. More particularly, the invention is directed to the provision of a fibrinogen-containing composition effective as a tissue adhesive, hemostat, or sealant.

Brief Summary Paragraph Right (3):
It is known that therapeutic compositions for use as tissue adhesives, sealants or hemostatic agents can be made using the proteins fibrinogen and thrombin, Cronkite, E. P. et al., J.A.M.A., 124, 976 (1944), Tidrick, R. T. and Warner, E. D., Surgery, 15, 90 (1944). Fibrinogen is a soluble protein found in the blood plasma of all vertebrates that when contacted by thrombin (another plasma protein) becomes polymerized to an insoluble gel-like network. In polymerized form, the fibrinogen is referred to as fibrin. The conversion of fibrinogen to fibrin is crucial to normal hemostatis in vertebrates.

Brief Summary Paragraph Right (10):
Broadly stated, this invention provides for a therapeutic fibrinogen composition effective as a tissue adhesive, hemostat, or sealant, and that is more effective, per concentration of fibrinogen contained therein, than presently available compositions. Accordingly, there is provided a therapeutic composition effective on contact with thrombin at a site of treatment in a patient as a tissue adhesive, hemostat or sealant, said composition comprising non-autologous, non-single donor mammalian fibrinogen that is capable of polymerizing when provided in solution at said site at a concentration of about 10 mg/ml thereof or less, to a fibrin network having therapeutically effective strength, and further comprising a sufficient amount of one or more physiologically-compatible solutes such that said composition, if formulated as a lyophilized material, can be reconstituted therefrom at room temperature in sterile water for injection in about 30 minutes or less, at about 25 mg/ml of said fibrinogen.

Brief Summary Paragraph Right (11):
There is provided also a therapeutic composition effective on contact with thrombin at a site of treatment in a patient as a tissue adhesive, hemostat or sealant, said composition comprising non-autologous, non-single donor mammalian fibrinogen that is capable of polymerizing when provided in solution at said site at a concentration of

about 30 mg/ml thereof or less, to a fibrin network having therapeutically effective strength, wherein said composition contains less than about 30% (w/w), based on total protein mass present therein, of proteins other than fibrinogen, and further comprises a sufficient amount of one or more low molecular weight physiologically-compatible solutes such that said composition, if formulated as a lyophilized material, can be reconstituted therefrom at room temperature in sterile water for injection in about 30 minutes or less, at about 25 mg/ml of said fibrinogen.

Detailed Description Paragraph Right (2):
This invention provides for fibrinogen-containing therapeutic compositions effective as tissue adhesives, tissue sealants or hemostatic agents. Although a therapeutic composition of the invention may perform only one of the above functions at a particular Site of treatment in a patient, all the compositions retain, nonetheless, the capacity to perform all three of the aforementioned functions. Additionally, there are clinical (medical) indications, such as the treatment of burns, plastic or reconstructive surgery, tissue grafting, or the treatment of anastomotic sites for which the compositions may perform simultaneously two or even all three of the aforementioned functions, that is, as adhesive, sealant and hemostatic agent.

Detailed Description Paragraph Right (26):
As aforementioned, it is preferred that at least about 80% of the fibrinogen in the therapeutic compositions of the invention be clottable. It is preferred also that the compositions contain less than about 30% (w/w) based on total protein mass present therein, of proteins other than fibrinogen. According to the practice of the invention (see Example 1 below), purification of fibrinogen may leave in contact therewith amounts of other protein species, notably serum albumin, gamma globulin, plasminogen, plasma fibronectin, and also factor XIII. Preferably, therapeutic fibrinogen compositions within the practice of the invention comprise, as percent by weight of total protein contained therein, clottable fibrinogen of at least about 56%, about 14% or less of non-clottable fibrinogen, serum albumin at less than about 20%, gamma globulin at less than about 10%, plasminogen at less than about 1%, and plasma fibronectin at less than about 3%. The concentration of serum albumin is most preferably less than about 4 to 5%.

Detailed Description Paragraph Right (27):
A highly preferred therapeutic composition which is produced routinely according to the process described in Example 1 below comprises (expressed as percent (w/w) of total protein contained therein) bovine fibrinogen at about 95%, and of which about 90% thereof is clottable; serum albumin at about 0.8%; gamma globulin at about 0.02%; plasma fibronectin at less than 0.5%; plasminogen at less than 0.02%; and also factor XIII at about 0.1 unit/mg fibrinogen.

Detailed Description Paragraph Right (74):
A representative analysis of the product derived from the above-described process was, as % (w/w) of total protein contained therein, fibrinogen 95%; serum albumin, 0.8%; gamma globulin, at 0.02%; plasma fibronectin, less than 0.5%; plasminogen, at less than 0.02%; and factor XIII, at about 1%, which equals an amount thereof such that if the lyophilized product were reconstituted to a solution having 20 to 25 mg/ml of fibrinogen, then the factor XIII would be present therein at about 0.1 unit/mg fibrinogen. Assays to determine the concentration of the copurifying proteins were performed as follows. For (bovine) fibrinogen, absorbance at 280 nm was determined based on a molar absorptivity of 1.55 ml/mg-cm at pH 7.0. Serum albumin, gamma globulin, plasma fibronectin, and plasminogen were each determined by ELISA methodology.

L15: Entry 39 of 56 File: USPT Jul 25, 1995

DOCUMENT-IDENTIFIER: US 5436136 A
TITLE: Repressible yeast promoters

Brief Summary Paragraph Right (39):
Example of such polypeptides are insulin, growth factors, such as epidermal, insulin-like, mast cell, nerve or transforming growth factor, growth hormones, such as human or bovine growth hormones, interleukin, such as interleukin-1 or -2, human macrophage migration inhibitory factor (MIF), interferons, such as human .alpha.-interferon, for example interferon-.alpha.A, .alpha.B, .alpha.D or .alpha.F, .beta.-interferon, .gamma.-interferon or a hybrid interferon, for example an .alpha.A-.alpha.D- or an .alpha.B-.alpha.D-hybrid interferon, hepatitis virus antigens, such as hepatitis B virus surface or core antigen or hepatitis A virus antigen, plasminogen activators, such as tissue plasminogen activator or urokinase, tumour necrosis factor, somatostatin, renin, .beta.-endorphin, immunoglobulins, such as the light and/or heavy chains of immunoglobulin D, E or G, immunoglobulin binding factors, such as immunoglobulin E binding factor, calcitonin, human calcitonin-related peptide, blood clotting factors, such as factor IX or VIIIc, eglin, such as eglin C, desulphatohirudin, such as desulphatohirudin variant HV1, HV2 or PA, or human superoxide dismutase. Preferred genes are those coding for a human .alpha.-interferon or hybrid interferon, human tissue plasminogen activator (t-PA), hepatitis B virus surface antigen (HBVsAg), insulin-like growth factor I, eglin C and desulphatohirudin variant HV1.

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L15: Entry 49 of 56 File: EPAB Dec 23, 1992

PUB-NO: WO009222309A1
DOCUMENT-IDENTIFIER: WO 9222309 A1
TITLE: USE OF 4-(4-CHLOROPHENYL-SULPHONYLCARBAMOYL) BENZOYL-L-VALYL-L-PROLINE 1(RS)-(1-TRIFLUOROACETYL-2-METHYLPROPYL) AMIDE IN THE TREATMENT OF VASCULAR DISEASES

PUBN-DATE: December 23, 1992

INVENTOR-INFORMATION:

NAME	COUNTRY
MEHTA, JAWEHAR LAL	US
SALDEEN, TOM GUSTAVE PER	SE
NICHOLS, WILMER WAYNE	US

ASSIGNEE-INFORMATION:

NAME	COUNTRY
ICI PLC	GB

APPL-NO: GB09201087
APPL-DATE: June 17, 1992

PRIORITY-DATA: GB09113164A (June 18, 1991)

INT-CL (IPC): A61K 37/02; A61K 37/64
EUR-CL (EPC): A61K038/05; A61K038/55

ABSTRACT:

CHG DATE=19990617 STATUS=O>The use of the elastase inhibitor 4-(4-chlorophenyl-sylphonylcarbamoyl)benzoyl-L-valyl-L-proline 1(RS)-(1-trifluoroacetyl-2-methylpropyl) amide, or a pharmaceutically acceptable salt thereof, in the treatment of certain vascular diseases in which neutrophils are involved, e.g. cardiovascular disease such as myocardial ischaemia, cerebrovascular disease such as stroke, peripheral vascular disease such as intermittent claudication, as well as in impaired reperfusion states such those associated with reconstructive vascular surgery.

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L15: Entry 45 of 56 File: USPT Jun 29, 1993

DOCUMENT-IDENTIFIER: US 5223409 A
TITLE: Directed evolution of novel binding proteins

Detailed Description Paragraph Right (376):
Serine proteases are an especially interesting class of potential target materials. Serine proteases are ubiquitous in living organisms and play vital roles in processes such as: digestion, blood clotting, fibrinolysis, immune response, fertilization, and post-translational processing of peptide hormones. Although the role these enzymes play is vital, uncontrolled or inappropriate proteolytic activity can be very damaging. Several serine proteases are directly involved in serious disease states. Uncontrolled neutrophil elastase (NE) (also known as leukocyte elastase) is thought to be the major cause of emphysema (BEIT86, HUBB86, HUBB89, HUTC87, SOMM90, WEWE87) whether caused by congenital lack of .alpha.-1-antitrypsin or by smoking. NE is also implicated as an essential ingredient in the pernicious cycle of: ##STR8## observed in cystic fibrosis (CF) (NADE90). Inappropriate NE activity is very harmful and to stop the progression of emphysema or to alleviate the symptoms of CF, an inhibitor of very high affinity is needed. The inhibitor must be very specific to NE lest it inhibit other vital serine proteases or esterases. Nadel (NADE90) has suggested that onset of excess secretion is initiated by 10.sup.-10 M NE; thus, the inhibitor must reduce the concentration of free NE to well below this level. Thus human neutrophil elastase is a preferred target and a highly stable protein is a preferred IPBD. In particular, BPTI, ITI-DI, or another BPTI homologue is a preferred IPBD for development of an inhibitor to HNE. Other preferred IPBDs for making an inhibitor to HNE include CMTI-III, SLPI, Eglin, .alpha.-conotoxin GI, and .OMEGA. Conotoxins.

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L15: Entry 14 of 56 File: USPT Nov 17, 1998

DOCUMENT-IDENTIFIER: US 5837500 A
TITLE: Directed evolution of novel binding proteins

Detailed Description Paragraph Right (381):
Serine proteases are an especially interesting class of potential target materials. Serine proteases are ubiquitous in living organisms and play vital roles in processes such as: digestion, blood clotting, fibrinolysis, immune response, fertilization, and post-translational processing of peptide hormones. Although the role these enzymes play is vital, uncontrolled or inappropriate proteolytic activity can be very damaging. Several serine proteases are directly involved in serious disease states. Uncontrolled neutrophil elastase (NE) (also known as leukocyte elastase) is thought to be the major cause of emphysema (BEIT86, HUBB86, HUBB89, HUTC87, SOMM90, WEWE87) whether caused by congenital lack of .alpha.-1-antitrypsin or by smoking. NE is also implicated as an essential ingredient in the pernicious cycle of: ##STR6## observed in cystic fibrosis (CF) (NADE90). Inappropriate NE activity is very harmful and to stop the progression of emphysema or to alleviate the symptoms of CF, an inhibitor of very high affinity is needed. The inhibitor must be very specific to NE lest it inhibit other vital serine proteases or esterases. Nadel (NADE90) has suggested that onset of excess secretion is initiated by 10.sup.-10 M NE; thus, the inhibitor must reduce the concentration of free NE to well below this level. Thus human neutrophil elastase is a preferred target and a highly stable protein is a preferred IPBD. In particular, BPTI, ITI-D1, or another BPTI homologue is a preferred IPBD for development of an inhibitor to HNE. Other preferred IPBDs for making an inhibitor to HNE include CMTI-III, SLPI, Eglin, .alpha.-conotoxin GI, and .OMEGA. Conotoxins.

L15: Entry 36 of 56 File: USPT Aug 29, 1995

DOCUMENT-IDENTIFIER: US 5446037 A
TITLE: 2-[(substituted) methylene]cephalosporin sulfones as antiinflammatory, antidegenerative and antithrombin agents

Brief Summary Paragraph Right (10):
The present inventors have discovered that 2-[(substituted)methylene]cephalosporin sulfones, particularly 2-[(heteroaryl substituted)methylene]cephalosporin sulfones are highly useful as elastase inhibitors and thus can be used in the treatment of inflammatory and degenerative diseases caused by proteolytic enzymes in mammals. The present inventors have also discovered that the compounds of the present invention are highly useful as anti-thrombin agents in the prevention, control and treatment of blood clotting.

Brief Summary Paragraph Right (12):
The present inventors have discovered that 2-[(substituted)methylene]cephalosporin sulfones and in particular 2-[heteroaryl(substituted)methylene]cephalosporin sulfones are potent elastase inhibitors useful in the prevention, control and treatment of inflammatory and degenerative conditions as well as potent anti-thrombin agents useful in the control, prevention and treatment of blood clotting.

L15: Entry 44 of 56 File: USPT Dec 21, 1993

DOCUMENT-IDENTIFIER: US 5271939 A
TITLE: Pharmaceutical compositions and methods of treatment to prevent and treat corneal scar formation produced by laser irradiation

Detailed Description Paragraph Right (36):
Epithelial cell health promoters as used herein, are compounds known to contribute to the health of the epithelial cells of the cornea. The presence of these compounds prior to, during, and/or after photoablation of the cornea can contribute to the prevention of corneal haze by encouraging the rapid resumption of epithelial integrity and prevention of stromal edema. Epithelial cell health promoters which can be used as adjuncts to the wound healing modulators of the present invention can include: ascorbic acid; retinoids, such as retinoic acid, retinol, retinal and retinoyl B-glucuronide; aloe vera; collagenase inhibitors; prostaglandins, such as prostaglandin E and elastase inhibitors.

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L6: Entry 17 of 39 File: USPT May 20, 1997

DOCUMENT-IDENTIFIER: US 5631011 A
TITLE: Tissue treatment composition comprising fibrin or fibrinogen and biodegradable and biocompatible polymer

Abstract Paragraph Right (1):
A tissue treatment composition, especially an adhesive composition comprises (i) fibrin or fibrinogen and (ii) a biodegradable and biocompatible polymer capable of forming a viscous aqueous solution. In addition to glueing, the tissue adhesive composition may be used for slow-release of a drug incorporated into it or for anti-adherence purposes, for wound healing, etc.

Brief Summary Paragraph Right (5):
To prevent a too early degradation of the fibrin clot by fibrinolys, the fibrin sealant composition may comprise a plasminogen activator inhibitor or a plasmin inhibitor, such as aprotinin. Such an inhibitor will also reduce the fibrinolytic activity resulting from any residual plasminogen in the fibrinogen composition.

Brief Summary Paragraph Right (8):
Fields of application include among others: ear, nose and throat surgery, general surgery, dentistry, neurosurgery, plastic surgery, thorax and vascular surgery, abdominal surgery, orthopaedics, accident surgery, gynaecology, urology, and opthalmology. Fibrin sealants have also been used for local application of drugs, such as antibiotics, growth factors and cytostatics. Commercial fibrin glues (prepared from human plasma) are available under the trade names Tissucol, TisseeI and Fibrin-Kleber Humano Immuno (Immuno AG, Vienna, Austria) as well as Beriplast (Behringwerke AG, Marburg, Germany) (these trade names being registered trademarks in several countries). Tisseel.TM. is a two-component kit containing a fluid thrombin component including calcium chloride and a somewhat more viscous fibrinogen component including factor XIII, fibronectin, aprotinin and plasminogen. The two components are delivered deep frozen in two separate syringes, or as two lyophilized powders with corresponding aprotinin and calcium solutions as solvents. As explained above the fibrin sealant consolidates when the two components are combined due to fibrin monomer aggregation. The setting rate is dependent on the thrombin concentration and varies from a few seconds (high thrombin concentration) to a couple of minutes (low thrombin concentration).

Detailed Description Paragraph Right (5):
Like the prior art fibrin sealants the tissue adhesive composition of the present invention may comprise additional constituents. Thus, in addition to sealer protein and viscosity enhancing polymer, such as e.g. high molecular polysaccharide, the composition will preferably comprise Factor XIII and/or fibronectin and/or plasminogen. Advantageously, the composition will also include clotting enzyme, i.e. thrombin, especially in combination with bivalent calcium, such as calcium chloride. The concentration of calcium chloride will then vary, e.g. between 40 mM to 0.2M depending on the specific purpose of the tissue adhesive composition, high concentrations of calcium chloride inhibiting fibroblast growth and therefore being preferred for anti-adherence applications (along with absence of fibronectin which stimulates the ingrowth of fibroblasts). It may further be valuable to include a fibrinolysis inhibitor, such as a plasmin inhibitor, e.g. aprotinin, aprilotinin, alpha-2-antiplasmin, alpha-2-macroglobulin, alpha-1-antitrypsin, epsilon-aminocaproic add or tranexamic acid, or a plasmin activator inhibitor, e.g. PAI-1 or PAI-2.

Detailed Description Paragraph Right (8):

The tissue treatment composition of the present invention may be presented in the same type of preparations as the prior art fibrin sealants. In an advantageous embodiment the tissue adhesive is therefore a two-component preparation, one component comprising the blood clot protein(s) and the other comprising thrombin and bivalent calcium as well as possible additives including fibrinolysis inhibitors. The viscosity enhancing polymer may be contained in one or both of the two components depending on the intended use of the tissue adhesive. While in the case of a fibrin glue the viscosity enhancing polymer may be contained in either or both of the two components, it is for other applications preferably associated with the fibrin or fibrinogen component. It is, of course, at least theoretically, also possible to provide the viscosity enhancing polymer as a separate component. The components may be provided in deep frozen solution form or as lyophilized powders, to be diluted prior to use with appropriate aqueous solutions, e.g. containing aprotinin and calcium ions, respectively.

Detailed Description Paragraph Right (25):
I. Original Tisseel.RTM. (Immuno AG, Austria) in which one syringe ("Fibrinogen component") contained 75-115 mg/ml of fibrinogen, 2-9 mg/ml of plasma fibronectin, 10-50 U of Factor XIII, 40-120 .mu.l of plasminogen and 3000 KIU/ml of aprotinin, and had a viscosity of about 100 cP. The other syringe ("Thrombin component") contained 500 IU/ml of thrombin and 40 mM CaCl.sub.2 and had a viscosity of 1.2 cP. The initial viscosity of the mixed contents (1+1) of the two syringes was well below 100 cP.

CLAIMS:

4. A tissue treatment composition according to claim 3 wherein wherein said protein which promotes wound healing is selected from the group consisting of fibronectin, aprotinin and plasminogen.

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L6: Entry 24 of 39 File: USPT May 23, 1995

DOCUMENT-IDENTIFIER: US 5418221 A
TITLE: Composition containing enamel matrix from tooth germs for inducing binding between living mineralized tissue parts

Brief Summary Paragraph Right (19):
According to a further preferred aspect of this invention the enamel matrix composition may be supplemented with a tissue adhesive based on fibrinogen, Factor XIII (which is a plasma-derived coagulation factor) and thrombin. Such supplemented composition may be constituted by a premix of enamel matrix and fibrinogen and Factor XIII, the thrombin being added immediately before applying the composition to the surgical site. The premix may optionally contain aprotinin to reduce the rate of decomposition. A preferred commercial product for use in such supplemented composition is Tisseel.RTM., a two-component fibrin sealant manufactured and sold by IMMUNO AG, Vienna, Austria.

Brief Summary Paragraph Right (20):
In using such tissue adhesive the premix of enamel matrix, fibrinogen, Factor XIII and, optionally, aprotinin, is mixed with a thrombin solution, and the resulting composition is then rapidly applied to the surgical site. In the treatment of periodontitis this technique greatly facilitates surgery. Thus, the adhesion of the composition to the root is enhanced, bleeding is stopped and positioning of the muco-periosteal flap is greatly simplified while eliminating the use of sutures.

Detailed Description Paragraph Right (13):
This example illustrates an improved method of application of "the attachment-promoting composition" in clinical practice. Patients with marginal periodontitis were treated as described in Example 5 with the only difference that a fibrin-based tissue adhesive was mixed in with the composition. The tissue adhesive is composed of fibrinogen, plasma-fibronectin, Factor XIII (a plasma-derived coagulation factor), plasminogen, aprotinin, thrombin and calcium chloride and manufactured by IMMUNO AG, Vienna, Austria. The tissue adhesive marketed under the name of Tisseel or Tissucol polymerizes after its various components have been mixed together to form an adhering coagulum. Thus, the addition of Tisseel (Tissucol) to "the attachment-promoting composition" enhances adhesion to the root surfaces during periodontal surgery. Furthermore, bleeding is stopped, facilitating visibility and positioning of the muco-periosteal flap. The flap can also be positioned more cervically on the root surfaces and sutures can be eliminated. Covering the root surfaces is essential for the healing results since this ultimately determines the degree of new attachment.

L6: Entry 28 of 39 File: USPT Mar 17, 1987

DOCUMENT-IDENTIFIER: US 4650678 A
TITLE: Readily dissolvable lyophilized fibrinogen formulation

Brief Summary Paragraph Right (8):
A fibrinogen lyophilizate, which is said to be suitable as a tissue adhesive, has been disclosed in German Offenlegungsschrift No. 3,002,934. This lyophilizate is also a cryoprecipitate, which accordingly contains not only fibrinogen and factor XIII, but also plasminogen, albumin and other plasma constituents. An inhibitor of plasminogen activator is added to stabilize the lyophilizate and the reconstituted solution. This lyophilizate is sparingly, and only at elevated temperature (37.degree. C.), soluble. After reconstitution, the product is stable for a maximum of 4 hours at room temperature.

Brief Summary Paragraph Right (9):
A lyophilized fibrinogen formulation has now been found, which formulation need not contain either an inhibitor of plasminogen activator or albumin to stabilize it and which is suitable for the preparation of highly concentrated fibrinogen solutions (about 8%) even at room temperature. It is unnecessary to employ cryoprecipitate as the starting point, since it is also possible to employ pure fibrinogen as the starting product.

Brief Summary Paragraph Right (22):
Since, when fibrinogen solutions of this type are used as tissue adhesives, the tenacity of adhesion increases with the content of fibrinogen, greater tenacity is obtained with tissue adhesives derived from concentrates prepared according to the invention.

Brief Summary Paragraph Right (24):
A further advantage of the fibrinogen concentrates prepared in the manner described is their stability after reconstitution. Since impurities of prothrombin factors and plasminogen are normally present in cryoprecipitates, the stability of cryoprecipitate solutions at room temperature is limited to 2 to 4 hours. Fibrinogen concentrates prepared according to the following Example 2 are, in contrast, stable at room temperature for a working day.

Searched for * fibrinogen + plasminogen

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L6: Entry 26 of 39 File: USPT Jul 19, 1994

DOCUMENT-IDENTIFIER: US 5330974 A
TITLE: Therapeutic fibrinogen compositions

Abstract Paragraph Left (1):
A therapeutic composition effective on contact with thrombin at a site of treatment in a patient as a tissue adhesive, hemostat or sealant, said composition comprising non-autologous, non-single donor mammalian fibrinogen that is capable of polymerizing when provided in solution at said site at a concentration of about 30 mg/ml thereof or less, to a fibrin network having therapeutically effective strength, wherein said composition contains less than about 30% (w/w), based on total protein mass present therein, of proteins other than fibrinogen, and further comprises a sufficient amount of one or more low molecular weight physiologically-compatible solutes such that said composition, if formulated as a lyophilized material, can be reconstituted therefrom at room temperature in sterile water for injection in about 30 minutes or less, at about 25 mg/ml of said fibrinogen. Additionally, methods for producing and maintaining said composition, and methods for the use thereof.

Brief Summary Paragraph Right (1):
This invention relates to therapeutic compositions comprising non-autologous non-single donor fibrinogen. More particularly, the invention is directed to the provision of a fibrinogen-containing composition effective as a tissue adhesive, hemostat, or sealant.

Brief Summary Paragraph Right (3):
It is known that therapeutic compositions for use as tissue adhesives, sealants or hemostatic agents can be made using the proteins fibrinogen and thrombin, Cronkite, E. P. et al., J.A.M.A., 124, 976 (1944), Tidrick, R. T. and Warner, E. D., Surgery, 15, 90 (1944). Fibrinogen is a soluble protein found in the blood plasma of all vertebrates that when contacted by thrombin (another plasma protein) becomes polymerized to an insoluble gel-like network. In polymerized form, the fibrinogen is referred to as fibrin. The conversion of fibrinogen to fibrin is crucial to normal hemostasis in vertebrates.

Brief Summary Paragraph Right (10):
Broadly stated, this invention provides for a therapeutic fibrinogen composition effective as a tissue adhesive, hemostat, or sealant, and that is more effective, per concentration of fibrinogen contained therein, than presently available compositions. Accordingly, there is provided a therapeutic composition effective on contact with thrombin at a site of treatment in a patient as a tissue adhesive, hemostat or sealant, said composition comprising non-autologous, non-single donor mammalian fibrinogen that is capable of polymerizing when provided in solution at said site at a concentration of about 10 mg/ml thereof or less, to a fibrin network having therapeutically effective strength, and further comprising a sufficient amount of one or more physiologically-compatible solutes such that said composition, if formulated as a lyophilized material, can be reconstituted therefrom at room temperature in sterile water for injection in about 30 minutes or less, at about 25 mg/ml of said fibrinogen.

Brief Summary Paragraph Right (11):
There is provided also a therapeutic composition effective on contact with thrombin at a site of treatment in a patient as a tissue adhesive, hemostat or sealant, said composition comprising non-autologous, non-single donor mammalian fibrinogen that is capable of polymerizing when provided in solution at said site at a concentration of

about 30 mg/ml thereof or less, to a fibrin network having therapeutically effective strength, wherein said composition contains less than about 30% (w/w), based on total protein mass present therein, of proteins other than fibrinogen, and further comprises a sufficient amount of one or more low molecular weight physiologically-compatible solutes such that said composition, if formulated as a lyophilized material, can be reconstituted therefrom at room temperature in sterile water for injection in about 30 minutes or less, at about 25 mg/ml of said fibrinogen.

Brief Summary Paragraph Right (17):
This invention provides for fibrinogen-containing therapeutic compositions effective as tissue adhesives, tissue sealants or hemostatic agents. Although a therapeutic composition of the invention may perform only one of the above functions at a particular site of treatment in a patient, all the compositions retain, nonetheless, the capacity to perform all three of the aforementioned functions. Additionally, there are clinical (medical) indications, such as the treatment of burns, plastic or reconstructive surgery, tissue grafting, or the treatment of anastomotic sites for which the compositions may perform simultaneously two or even all three of the aforementioned functions, that is, as adhesive, sealant and hemostatic agent.

Brief Summary Paragraph Right (40):
As aforementioned, it is preferred that at least about 80% of the fibrinogen in the therapeutic compositions of the invention be clottable. It is preferred also that the compositions contain less than about 30% (w/w) based on total protein mass present therein, of proteins other than fibrinogen. According to the practice of the invention (see Example 1 below), purification of fibrinogen may leave in contact therewith amounts of other protein species, notably serum albumin, gamma globulin, plasminogen, plasma fibronectin, and also factor XIII. Preferably, therapeutic fibrinogen compositions within the practice of the invention comprise, as percent by weight of total protein contained therein, clottable fibrinogen of at least about 56%, about 14% or less of non-clottable fibrinogen, serum albumin at less than about 20%, gamma globulin at less than about 10%, plasminogen at less than about 1%, and plasma fibronectin at less than about 3%. The concentration of serum albumin is most preferably less than about 4 to 5%.

Brief Summary Paragraph Right (41):
A highly preferred therapeutic composition which is produced routinely according to the process described in Example 1 below comprises (expressed as percent (w/w) of total protein contained therein) bovine fibrinogen at about 95% and of which about 90% thereof is clottable; serum albumin at about 0.8%; gamma globulin at about 0.02%; plasma fibronectin at less than 0.5%; plasminogen at less than 0.02%; and also factor XIII at about 0.1 unit/mg fibrinogen.

Detailed Description Paragraph Right (10):
A representative analysis of the product derived from the above-described process was, as % (w/w) of total protein contained therein, fibrinogen 95%; serum albumin, 0.8%; gamma globulin, at 0.02%; plasma fibronectin, less than 0.5%; plasminogen, at less than 0.02%; and factor XIII, at about 1%, which equals an amount thereof such that if the lyophilized product were reconstituted to a solution having 20 to 25 mg/ml of fibrinogen, then the factor XIII would be present therein at about 0.1 unit/mg fibrinogen. Assays to determine the concentration of the copurifying proteins were performed as follows. For (bovine) fibrinogen, absorbance at 280 nm was determined based on a molar absorptivity of 1.55 ml/mg-cm at pH 7.0. Serum albumin, gamma globulin, plasma fibronectin, and plasminogen were each determined by ELISA methodology.

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L1: Entry 12 of 13 File: USPT Apr 19, 1994

DOCUMENT-IDENTIFIER: US 5304482 A
TITLE: Serine protease mutants of the chymotrypsin superfamily resistant to inhibition by their cognate inhibitors

Brief Summary Paragraph Right (5):
Examples of serine proteases of the chymotrypsin superfamily include tissue-type plasminogen activator (hereinafter "t-PA"), trypsin, trypsin-like protease, chymotrypsin, plasmin, elastase, urokinase (or urinary-type plasminogen activator (hereinafter "u-PA")), acrosin, activated protein C, C1 esterase, cathepsin G, chymase and proteases of the blood coagulation cascade including kallikrein, thrombin, and Factors VIIa, IXa, Xa, XIa and XIIa (Barrett, A.J., In: Proteinase Inhibitors, Ed. Barrett, A.J. et al, Elsevier, Amsterdam, pages 3-22 (1986); Strassburger, W. et al, FEBS Lett., 157:219-223 (1983); Dayhoff, M.O., Atlas of Protein Sequence and Structure, Vol. 5, National Biomedical Research Foundation, Silver Spring, Maryland (1972); and Rosenberg, R.D. et al, Hosp. Prac., 21:131-137 (1986)).

Brief Summary Paragraph Right (26):
Serine protease inhibitors belonging to the serpin family include the plasminogen activator inhibitors PAI-1, PAI-2 and PAI-3, C1 esterase inhibitor, alpha-2-antiplasmin, contrapsin, alpha-1-antitrypsin, antithrombin III, protease nexin I, alpha-1-antichymotrypsin, protein C inhibitor, heparin cofactor II and growth hormone regulated protein (Carrell, R.W. et al, Cold Spring Harbor Symp. Quant. Biol., 52:527-535 (1987); Sommer, J. et al, Biochem., 26:6407-6410 (1987); Suzuki, K. et al, J. Biol. Chem., 262:611-616 (1987); and Stump, D.C. et al, J. Biol. Chem., 261:12759-12766 (1986)).

Brief Summary Paragraph Right (40):
A particularly important serine protease of the chymotrypsin superfamily is t-PA. Most members of the chymotrypsin family of serine proteases are synthesized as inactive, single chain precursors or zymogens. Subsequent cleavage of a specific peptide bond converts these precursors into fully active two-chain enzymes. By contrast, the single-chain form of t-PA displays significant catalytic activity and its V.sub.max for generation of plasmin from plasminogen is only about 3-5 fold lower than that of two-chain t-PA (Boose, J. A. et al, Biochem., 28:635-643 (1988); and Petersen, L.C. et al, Biochim. Biophys. Acta, 952:245-254 (1988)).

Brief Summary Paragraph Right (41):
t-PA is currently being used, via intracoronary or intravenous administration, to treat myocardial infarction, pulmonary embolism, and deep venous thrombosis, although it does not work directly to dissolve thrombi (blood clots). Rather, t-PA promotes cleavage of the peptide bond between Arg.sub.560 and Val.sub.561 of plasminogen (Robbins, K.C. et al, J. Biol. Chem., 242:2333-2342 (1967)), thereby converting the inactive zymogen into the powerful but non-specific protease, plasmin, which then degrades the fibrin mesh work of the blood clot (Bachmann, F. et al, Semin. Throm. Haemost., 43:77-89 (1984); Gerard, R.D. et al, Mol. Biol. Med., 3:449-557 (1986); and Verstraete, M. et al, Blood, 67:1529-1541 (1986)).

Brief Summary Paragraph Right (42):
t-PA produces local fibrinolysis without necessarily depleting systemic fibrinogen. This is because t-PA has the ability to bind directly to fibrin, forming a fibrin-t-PA complex whose affinity for plasminogen is increased approximately 500 fold (Ranby, M. et al, Biochim. Biophys. Acta, 704:461-469 (1982); and Rijken, D.C. et al, J. Biol. Chem., 257:2920-2925 (1982)). Thus, binding of intravenously-administered t-PA to

* Searched for: Plasminogen fibrinogen + elastase inhibitors

coronary thrombi, where plasminogen is also present in high concentration (Wiman, B. et al, Nature, 272:549-550 (1978)), results in efficient production of plasmin at the site of the thrombus where it will do the most good.

Brief Summary Paragraph Table (5):
TABLE V Serine Protease Potato Inhibitor
Chymotrypsin Barley chymotrypsin inhibitor
Subtilisin Novo Barley chymotrypsin inhibitor Subtilisin Carlsberg Leech inhibitor
eglin

Drawing Description Paragraph Right (37):
The enzymatic activity of the single-chain form of t-PA is probably responsible for the 30-50% depletion of circulating fibrinogen that occurs in many patients receiving the drug (Collen, D. et al, Circ., 73:511-517 (1986); and Rao, A.K. et al, J. Am. Coll. Cardiol., 11:1-11 (1988)) and perhaps for the hemorrhagic complications that occur in a very small minority (Califf, R.M. et al, Am. J. Med., 85:353-359 (1988)). Thus, in the present invention, the possibility of reducing these problems by generating variants of t-PA whose catalytic activity in the single-chain form is greatly reduced has been explored. Moreover, these variants of t-PA, while in the single-chain form, have been demonstrated in the present invention to exhibit reduced reactivity towards cognate inhibitors. Once attached to the fibrin meshwork of a thrombus, however, such variants are expected in the present invention to be cleaved by plasmin generated locally by the subject's own t-PA, and thus then display full catalytic activity.

Drawing Description Paragraph Left (1):
Second, t-PA carries an additional stretch of seven amino acids (.sub.296 KHRRSPG.sub.302, see FIG. 1) located adjacent to predicted contact between t-PA(R.sub.304) and PAI-1(E.sub.350). Four out of seven of these amino acids are positively-charged, while the predicted complementary region of PAI-1(.sub.350 EEIIMD.sub.355) contains three negatively-charged residues. It was believed in the present invention that electrostatic interactions between these regions may play an important role in the formation or stabilization of complexes between t-PA and PAI-1. By contrast, such interactions could not occur when t-PA interacts with its substrate, plasminogen (PLG), which has no negatively-charged residues in the equivalent region (see Table VII above).

Detailed Description Paragraph Right (27):
An indirect chromogenic assay was carried out so as to determine the activities of the wild-type and mutant t-PAs produced in the COS cells. In this assay, free p-nitroaniline is released from the chromogenic substrate Spectrozyme PL (H-D-norleucylhexahydrotyrosyl-lysine-p-nitroanilide diacetate salt) (American Diagnostica, Inc.) by the action of plasmin generated by the action of t-PA on plasminogen. The release of free p-nitroaniline was measured spectrophotometrically at OD.sub.405 nm.

Detailed Description Paragraph Right (28):
More specifically, 100 .mu.l reaction mixtures comprising 150-200 pg of the t-PA to be tested, 0.4 mM of Spectrozyme PL, 0.1 .mu.M of Lys-plasminogen (American Diagnostica, Inc.) and 0.5-25 .mu.g/ml of soluble fibrin (Des-A-fibrinogen) (American Diagnostica, Inc.) in a buffer comprising 50 mM Tris-HCl (pH 7.5), 0.1M NaCl, 1.0 mM EDTA and 0.01% (v/v) Tween 80 were incubated at 37.degree. C. in 96-Well, flat-bottomed microtiter plates (Costar, Inc.) and the OD.sub.405 nm was measured with a Bio-tek microplate reader (Model EL310) at 15 or 30 minute intervals over a 2 hour period. Aliquots of buffer or appropriately-diluted samples of medium from mock-transfected cells were analyzed as controls and the OD values obtained (<0.01 unit) were subtracted from the corresponding test values. Delta OD values were measured as the change in optical density between 30 minutes and 60 minutes, i.e., following the lag phase of the reaction and the complete conversion of single-chain t-PA to the two-chain form. Under the conditions used in the standard assay (0.1 .mu.M of Lys-plasminogen and 25 .mu.g/ml of Des-A-fibrinogen), soluble fibrin stimulated the activity of t-PA 20-40 fold. The results are shown in FIG. 4.

Detailed Description Paragraph Right (29):
As shown in FIG. 4, all three of the above-described t-PA mutants of the present

invention were found to be enzymatically active and their specific activities were not found to be significantly different from that of wild-type t-PA. In addition, the above-described t-PA mutants of the present invention were found to respond to varying concentrations of Des-A-fibrinogen in a manner similar to that of wild-type t-PA. The maximal stimulation by Des-A-fibrinogen was 20-40 fold. This is in agreement with the observations of others on wild-type t-PA using a Des-A-fibrinogen preparation (Karlan, B. et al, Biochem. Biophys. Res. Comm., 142:147-154 (1987)). In each case, half-maximal stimulation occurred when Des-A-fibrinogen was present at a concentration of approximately 1.0 .mu.g/ml.

Detailed Description Paragraph Right (30):
Next, the K.sub.m and K.sub.cat values of the wild-type and mutant t-PAs were determined by assaying the various forms of the enzyme in the presence of saturating concentrations of Des-A-fibrinogen (25 .mu.g/ml) and different concentrations (from 0.02-0.16 .mu.M) of the substrate, Lys-plasminogen. The results are shown in Table X below.

Detailed Description Paragraph Right (32):
The data shown in FIG. 4 and Table X demonstrate that (i) deletion of amino acids 296-302 of t-PA and (ii) substitution of Ser or Glu for Arg at position 304 have little effect on the ability of t-PA to activate plasminogen and to be stimulated by soluble fibrin fragments.

Detailed Description Paragraph Right (35):
The above data indicate that amino acids 296-302 and 304 are not involved in catalytic functions of t-PA, but play an important role in the interaction of the enzyme with its cognate serine protease inhibitor, PAI-1. Using the structure of trypsin as a model, these amino acids are predicted to map near the active site of the serine protease, some distance from the catalytic triad. Thus, the area of contact between t-PA and PAI-1 is more extensive than the interaction between t-PA and its true substrate plasminogen.

Detailed Description Paragraph Right (39):
The data presented in Section F. above demonstrate that residues 296-302 and 304 of t-PA play an important role in interaction of the enzyme with the cognate inhibitor, PAI-1, but not with the substrate, Lys-plasminogen. Modeling of the catalytic domain of t-PA based on the known structure of trypsin suggests that residues 296-302 form a surface loop at the edge of the enzyme's active site. This loop is highly positively charged. As discussed above in Sections A and F, it has been proposed in the present invention that the effect of this region may be mediated by its formation of electrostatic bonds with PAI-1. To test this hypothesis, each of the charged residues within the loop were altered and the effect of these mutations upon the enzyme's interaction with PAI-1 was assessed as described below. If the positively charged residues in the loop form salt bridges with a complementary region of the serine protease inhibitor, PAI-1, then their substitution by negatively charged residues would be expected to be disruptive of interactions between t-PA and PAI-1 due to the juxtaposition of the side chains of similarly charged residues during the association of these two proteins.

Detailed Description Paragraph Right (53):
Next, the K.sub.m and K.sub.cat values of the wild-type and mutant t-PAs were determined by assaying the various forms of the enzyme in the presence of saturating concentrations of Des-A-fibrinogen (25 .mu.g/ml) and different concentrations (from 0.02-0.16 .mu.M) of the substrate, Lys-plasminogen. The results are shown in Table XI below.

Detailed Description Paragraph Right (55):
Similarly, the data presented in FIG. 6 suggests that the mutations have not altered t-PA's interaction with its positive effector, Des-A-fibrinogen. By contrast, the data presented in FIG. 7 indicates clear differences in the behavior of wild-type t-PA and some of the mutant t-PAs. Specifically, the ability to interact normally with the serpin, PAI-1, has been substantially changed for three of the mutant t-PAs, i.e., t-PA(R.sub.298 .fwdarw.E), t-PA(R.sub.299 .fwdarw.E), and t-PA(K.sub.296, R.sub.298, R.sub.299 .fwdarw.E, E, E). The behavior of the triple mutant is particularly striking; even after pre-incubation with a greater than 200-fold molar excess of

PAI-1, the triple mutant shows no loss of activity. These findings support the proposal that the surface loop of t-PA, i.e., residues 296-302, interacts specifically with the cognate inhibitor, PAI-1, and suggest that this interaction involves Arg.sub.298 and Arg.sub.299. These observations are consistent with the hypothesis that the specific interactions between t-PA and PAI-1 involve electrostatic bonds. The residues of t-PA involved in these interactions are Arg.sub.298, Arg.sub.299, and Arg.sub.304.

Detailed Description Paragraph Right (82):
The rate of inhibition of 60 pM t-PA was studied under pseudo-first order conditions using inhibitor concentrations ranging from 0.6 to 100 nM. The t-PA-PAI-1 mixes were preincubated in microtiter plate wells at 24.degree. C. for various time periods (from 0 to 30 minutes) before the addition of a mixture of Lys-plasminogen, Spectrozyme PL, and Des-A-fibrinogen to final concentrations of 300 nM, 0.4 nM, and 12.5 .mu.g/ml, respectively. After the addition of substrates, the microtiter plates were incubated at 37.degree. C. and the absorbance at 405 nm was monitored for 2 hours to determine the residual t-PA activity.

L1: Entry 10 of 13 File: USPT Dec 5, 1995

DOCUMENT-IDENTIFIER: US 5472942 A
TITLE: Anti-thrombins

Brief Summary Paragraph Right (10):
The leech-derived anti-thrombin according to the invention (and corresponding DNA sequences which can be extrapolated therefrom) is non-homologous with eglin, a known elastase/chymotrypsin inhibitor which is known to be present in the medicinal leech Hirudo medicinails, and is described by Seemuller et al in "Eglin: elastase-cathepsin G inhibitor from leeches"; 1981 Meth. Enzymol. 80: pp.804-816.

Detailed Description Paragraph Right (1):
The anti-thrombin according to the invention may be used in a pharmaceutical formulation, together with a pharmaceutically acceptable carrier or excipient therefor. Such a formulation is typically for intravenous administration (in which case the carrier is generally sterile saline or water of acceptable purity). The anti-thrombin according to the invention is suitable for treatment of thromboembolic events, such as the coagulation of blood. In one embodiment of the invention, the anti-thrombin is coadministered with a plasminogen activator, such as tissue plasminogen activator; the anti-thrombin according to the invention has been found to be compatible with the latter.

Detailed Description Paragraph Right (36):
Antithrombin activity was determined by measuring the inhibitor of the clotting activity of thrombin upon fibrinogen essentially as described by Markwardt in Methods in Enzymology; XIX, pp924; "Hirudin as an inhibitor of Thrombin" (1970), or by measuring the inhibition of thrombin cleavage of specific para-nitrophenol derived chromogenic substrates such as S-238 (commercially available from Kabi).

CLAIMS:

11. A medicament for according to claim 10, further comprising a plasminogen activator.

16. A composition comprising the polypeptide according to claim 12 and a plasminogen activator.

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L1: Entry 9 of 13 File: USPT Jan 23, 1996

DOCUMENT-IDENTIFIER: US 5486602 A
TITLE: Genes encoding serine protease mutants of the chymotrypsin superfamily resistant to inhibition by their cognate inhibitors

Brief Summary Paragraph Right (4):
Examples of serine proteases of the chymotrypsin superfamily include tissue-type plasminogen activator (hereinafter "t-PA"), trypsin, trypsin-like protease, chymotrypsin, plasmin, elastase, urokinase (or urinary-type plasminogen activator (hereinafter "u-PA")), acrosin, activated protein C, Cl esterase, cathepsin G, chymase and proteases of the blood coagulation cascade including kallikrein, thrombin, and Factors VIIa, IXa, Xa, XIa and XIIa (Barrett, A. J., In: Proteinase Inhibitors, Ed. Barrett, A. J. et al, Elsevier, Amsterdam, pages 3-22 (1986); Strassburger, W. et al, FEBS Lett., 157:219-223 (1983); Dayhoff, M. O., Atlas of Protein Sequence and Structure, Vol. 5, National Biomedical Research Foundation, Silver Spring, Md. (1972); and Rosenberg, R. D. et al, Hosp. Prac., 21:131-137 (1986)).

Brief Summary Paragraph Right (14):
Serine protease inhibitors belonging to the serpin family include the plasminogen activator inhibitors PAI-1, PAI-2 and PAI-3, Cl esterase inhibitor, alpha-2-antiplasmin, contrapsin, alpha-1-antitrypsin, antithrombin III, protease nexin I, alpha-1-antichymotrypsin, protein C inhibitor, heparin cofactor II and growth hormone regulated protein (Carrell, R. W. et al, Cold Spring Harbor Symp. Quant. Biol., 52:527-535 (1987); Sommer, J. et al, Biochem, 26:6407-6410 (1987); Suzuki, K. et al, J. Biol. Chem., 611-616 (1987); and Stump, D. C. et al, J. Biol. Chem., 261:12759-12766 (1986)).

Brief Summary Paragraph Right (23):
A particularly important serine protease of the chymotrypsin superfamily is t-PA. Most members of the chymotrypsin family of serine proteases are synthesized as inactive, single chain precursors or zymogens. Subsequent cleavage of a specific peptide bond converts these precursors into fully active two-chain enzymes. By contrast, the single-chain form of t-PA displays significant catalytic activity and its V.sub.max for generation of plasmin from plasminogen is only about 3-5 fold lower than that of two-chain t-PA (Boose, J. A. et al, Biochem., 28:635-643 (1988); and Petersen, L. C. et al, Biochim., Biophys. Acta 952:245-254 (1988)) .

Brief Summary Paragraph Right (24):
t-PA is currently being used, via intracoronary or intravenous administration, to treat myocardial infarction, pulmonary embolism, and deep venous thrombosis, although it does not work directly to dissolve thrombi (blood clots). Rather, t-PA promotes cleavage of the peptide bond between Arg.sub.560 and Val.sub.516 of plasminogen (Robbins, K. C. et al, J. Biol. Chem., 242:2333-2342 (1967)), thereby converting the inactive zymogen into the powerful but non-specific protease, plasmin, which then degrades the fibrin mesh work of the blood clot (Bachmann, F. et al, Semin. Throm. Haemost., 43:77-89 (1984); Gerard, R. D. et al, Mol. Biol. Med., 3:449-557 (1986); and Verstraete, M. et al, Blood, 67:1529-1541 (1986)).

Brief Summary Paragraph Right (25):
t-PA produces local fibrinolysis without necessarily depleting systemic fibrinogen. This is because t-PA has the ability to bind directly to fibrin, forming a fibrin-t-PA complex whose affinity for plasminogen is increased approximately 500 fold (Ranby, M. et al, Biochim. Biophys. Acta, 704:461-469 (1982); and Rijken, D. C. et al, J. Biol. Chem., 257:2920-2925 (1982)). Thus, binding of intravenously-administered t-PA to

coronary thrombi, where plasminogen is also present in high concentration (Wiman, B. et al, Nature, 272:549-550 (1978)), results in efficient production of plasmin at the site of the thrombus where it will do the most good.

Brief Summary Paragraph Table (5):
TABLE V _____ Serine Protease Potato Inhibitor
_____ Chymotrypsin Barley chymotrypsin inhibitor
Subtilism Novo Barley chymotrypsin inhibitor Subtilism Carlsberg Leech inhibitor eglin
_____

Detailed Description Paragraph Right (25):
The enzymatic activity of the single-chain form of t-PA is probably responsible for the 30-50% depletion of circulating fibrinogen that occurs in many patients receiving the drug (Collen, D. et al, Circ., 75:511-517 (1986); and Rao, A. K. et al, J. Am. Coll. Cardiol., 11:1-11 (1988)) and perhaps for the hemorrhagic complications that occur in a very small minority (Califf, R. M. et al, Am. J. Med., 85:353-359 (1988)). Thus, in the present invention, the possibility of reducing these problems by generating variants of t-PA whose catalytic activity in the single-chain form is greatly reduced has been explored. Moreover, these variants of t-PA, while in the single-chain form, have been demonstrated in the present invention to exhibit reduced reactivity towards cognate inhibitors. Once attached to the fibrin meshwork of a thrombus, however, such variants are expected in the present invention to be cleaved by plasmin generated locally by the subject's own t-PA, and thus then display full catalytic activity.

Detailed Description Paragraph Right (55):
An indirect chromogenic assay was carried out so as to determine the activities of the wild-type and mutant t-PAs produced in the COS cells. In this assay, free p-nitroaniline is released from the chromogenic substrate Spectrozyme PL (H-D-norleucylhexahydrotyrosyl-lysine-p-nitroanilide diacetate salt) (American Diagnostica, Inc.) by the action of plasmin generated by the action of t-PA on plasminogen. The release of free p-nitroaniline was measured spectrophotometrically at OD.sub.405 nm.

Detailed Description Paragraph Right (56):
More specifically, 100 .mu.l reaction mixtures comprising 150-200 pg of the t-PA to be tested, 0.4 mM of Spectrozyme PL, 0.1 .mu.M of Lys-plasminogen (American Diagnostica, Inc.) and 0.5-25 .mu.g/ml of soluble fibrin (Des-A-fibrinogen) (American Diagnostica, Inc.) in a buffer comprising 50 mM Tris-HCl (pH 7.5), 0.1M NaCl, 1.0 mM EDTA and 0.01% (v/v) Tween 80 were incubated at 7.degree. C in 96-well, flat-bottomed microtiter plates (Costar, Inc.) and the OD.sub.405 nm was measured with a Bio-tek microplate reader (Model EL310) at 15 or 30 minute intervals over a 2 hour period. Aliquots of buffer or appropriately-diluted samples of medium from mock-transfected cells were analyzed as controls and the OD values obtained (<0.01 unit) were subtracted from the corresponding test values. Delta OD values were measured as the change in optical density between 30 minutes and 60 minutes, i.e., following the lag phase of the reaction and the complete conversion of single-chain t-PA to the two-chain form. Under the conditions used in the standard assay (0.1 .mu.M of Lys-plasminogen and 25 .mu.g/ml of Des-A-fibrinogen), soluble fibrin stimulated the activity of t-PA 20-40 fold. The results are shown in FIG. 4.

Detailed Description Paragraph Right (57):
As shown in FIG. 4, all three of the above-described t-PA mutants of the present invention were found to be enzymatically active and their specific activities were not found to be significantly different from that of wild-type t-PA. In addition, the above-described t-PA mutants of the present invention were found to respond to varying concentrations of Des-A-fibrinogen in a manner similar to that of wild-type t-PA. The maximal stimulation by Des-A-fibrinogen was 20-40 fold. This is in agreement with the observations of others on wild-type t-PA using a Des-A-fibrinogen preparation (Karlan, B. et al, Biochem. Biophys. Res. Comm., 142:147-154 (1987)). In each case, half-maximal stimulation occurred when Des-A-fibrinogen was present at a concentration of approximately 1.0 .mu.g/ml.

Detailed Description Paragraph Right (58):
Next, the K.sub.m and K.sub.cat values of the wild-type and mutant t-PAs were

determined by assaying the various forms of the enzyme in the presence of saturating concentrations of Des-A-fibrinogen (25 .mu.g/ml) and different concentrations (from 0.02-0.16 .mu.M) of the substrate, Lys-plasminogen. The results are shown in Table X below.

Detailed Description Paragraph Right (60):
The data shown in FIG. 4 and Table X demonstrate that (i) deletion of amino acids 296-302 of t-PA and (ii) substitution of Ser or Glu for Arg at position 304 have little effect on the ability of t-PA to activate plasminogen and to be stimulated by soluble fibrin fragments.

Detailed Description Paragraph Right (63):
The above data indicate that amino acids 296-302 and 304 are not involved in catalytic functions of t-PA, but play an important role in the interaction of the enzyme with its cognate serine protease inhibitor, PAI-1. Using the structure of trypsin as a model, these amino acids are predicted to map near the active site of the serine protease, some distance from the catalytic triad. Thus, the area of contact between t-PA and PAI-1 is more extensive than the interaction between t-PA and its true substrate plasminogen.

Detailed Description Paragraph Right (66):
The data presented in Section F. above demonstrate that residues 296-302 and 304 of t-PA play an important role in interaction of the enzyme with the cognate inhibitor, PAI-1, but not with the substrate, Lys-plasminogen. Modeling of the catalytic domain of t-PA based on the known structure of trypsin suggests that residues 296-302 form a surface loop at the edge of the enzyme's active site. This loop is highly positively charged. As discussed above in Sections A and F, it has been proposed in the present invention that the effect of this region may be mediated by its formation of electrostatic bonds with PAI-1. To test this hypothesis, each of the charged residues within the loop were altered and the effect of these mutations upon the enzyme's interaction with PAI-1 was assessed as described below. If the positively charged residues in the loop form salt bridges with a complementary region of the serine protease inhibitor, PAI-1, then their substitution by negatively charged residues would be expected to be disruptive of interactions between t-PA and PAI-1 due to the juxtaposition of the side chains of similarly charged residues during the association of these two proteins.

Detailed Description Paragraph Right (77):
Next, the K.sub.m and K.sub.cat values of the wild-type and mutant t-PAs were determined by assaying the various forms of the enzyme in the presence of saturating concentrations of Des-A-fibrinogen (25 .mu.g/ml) and different concentrations (from 0.02-0.16 .mu.M) of the substrate, Lys-plasminogen. The results are shown in Table XI below.

Detailed Description Paragraph Right (79):
Similarly, the data presented in FIG. 6 suggests that the mutations have not altered t-PA's interaction with its positive effector, Des-A-fibrinogen. By contrast, the data presented in FIG. 7 indicates clear differences in the behavior of wild-type t-PA and some of the mutant t-PAs. Specifically, the ability to interact normally with the serpin, PAI-1, has been substantially changed for three of the mutant t-PAs, i.e., t-PA(R.sub.298 .fwdarw.E), t-PA(R.sub.299 .fwdarw.E), and t-PA(K.sub.296, R.sub.298, R.sub.299 .fwdarw.E, E, E). The behavior of the triple mutant is particularly striking; even after pre-incubation with a greater than 200-fold molar excess of PAI-1, the triple mutant shows no loss of activity. These findings support the proposal that the surface loop of t-PA, i.e., residues 296-302, interacts specifically with the cognate inhibitor, PAI-1, and suggest that this interaction involves Arg.sub.298 and Arg.sub.299. These observations are consistent with the hypothesis that the specific interactions between t-PA and PAI-1 involve electrostatic bonds. The residues of t-PA involved in these interactions are Arg.sub.298, Arg.sub.299, and Arg.sub.304.

Detailed Description Paragraph Right (102):
The rate of inhibition of 60 pM t-PA was studied under pseudo-first order conditions using inhibitor concentrations ranging from 0.6 to 100 nM. The t-PA-PAI-1 mixes were preincubated in microtiter plate wells at 24.degree. C. for various time periods (from

0 to 30 minutes) before the addition of a mixture of Lys-plasminogen, Spectrozyme PL, and Des-A-fibrinogen to final concentrations of 300 nM, 0.4 nM, and 12.5 .mu.g/ml, respectively. After the addition of substrates, the microtiter plates were incubated at 37.degree. C. and the absorbance at 405 nm was monitored for 2 hours to determine the residual t-PA activity.

Detailed Description Paragraph Left (1):
Second, t-PA carries an additional stretch of seven amino acids (.sub.296 KHRRSPG.sub.302, see FIG. 1) located adjacent to predicted contact between t-PA(R.sub.304) and PAI-1(E.sub.50). Four out of seven of these amino acids are positively-charged, while the predicted complementary region of PAI-1(.sub.350 EEIIMD.sub.355) contains three negatively-charged residues. It was believed in the present invention that electrostatic interactions between these regions may play an important role in the formation or stabilization of complexes between t-PA and PAI-1. By contrast, such interactions could not occur when t-PA interacts with its substrate, plasminogen (PLG), which has no negatively-charged residues in the equivalent region (see Table VII above).

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L1: Entry 7 of 13 File: USPT Nov 5, 1996

DOCUMENT-IDENTIFIER: US 5571698 A
TITLE: Directed evolution of novel binding proteins

Detailed Description Paragraph Right (404):
Serine proteases are an especially interesting class of potential target materials. Serine proteases are ubiquitous in living organisms and play vital roles in processes such as: digestion, blood clotting, fibrinolysis, immune response, fertilization, and post-translational processing of peptide hormones. Although the role these enzymes play is vital, uncontrolled or inappropriate proteolytic activity can be very damaging. Several serine proteases are directly involved in serious disease states. Uncontrolled neutrophil elastase (NE) (also known as leukocyte elastase) is thought to be the major cause of emphysema (BEIT86, HUBB86, HUBB89, HUTC87, SOMM90, WEWE87) whether caused by congenital lack of .alpha.-1-antitrypsin or by smoking. NE is also implicated as an essential ingredient in the pernicious cycle of: ##STR7## observed in cystic fibrosis (CF) (NADE90). Inappropriate NE activity is very harmful and to stop the progression of emphysema or to alleviate the symptoms of CF, an inhibitor of very high affinity is needed. The inhibitor must be very specific to NE lest it inhibit other vital serine proteases or esterases. Nadel (NADE90) has suggested that onset of excess secretion is initiated by 10.sup.-10 M NE; thus, the inhibitor must reduce the concentration of free NE to well below this level. Thus human neutrophil elastase is a preferred target and a highly stable protein is a preferred IPBD. In particular, BPTI, ITI-D1, or another BPTI homologue is a preferred IPBD for development of an inhibitor to HNE. Other preferred IPBDs for making an inhibitor to HNE include CMTI-III, SLPI, Eglin, .alpha.-conotoxin GI, and .OMEGA. Conotoxins.

Detailed Description Paragraph Right (1101):
MCPH85: McPhalen, C A, H P Schnebli, and M N G James, "Crystal and molecular structure of the inhibitor eglin from leeches in complex with subtilisin Carlsberg", FEBS Lett (1985), 188(1)55-8.

Detailed Description Paragraph Left (24):
Optionally, DNA encoding a flexible linker of one to 10 amino acids is introduced between the ipbd gene fragment and the Pf3 coat-protein gene. Optionally, DNA encoding the recognition site for a specific protease, such as tissue plasminogen activator or blood clotting Factor Xa, is introduced between the ipbd gene fragment and the Pf3 coat-protein gene. Amino acids that form the recognition site for a specific protease may also serve the function of a flexible linker. This tripartite gene is introduced into Pf3 so that it does not interfere with expression of any Pf3 genes. To reduce the possibility of genetic recombination, part (3) is designed to have numerous silent mutations relative to the wild-type gene. Once the signal sequence is cleaved off, the IPBD is in the periplasm and the mature coat protein acts as an anchor and phage-assembly signal. It matters not that this fusion protein comes to rest in the lipid bilayer by a route different from the route followed by the wild-type coat protein.

Detailed Description Paragraph Type 1 (205):
9) fibrinogen

Detailed Description Paragraph Table (56):
TABLE 50

Number	Amino Acids	Cross Structure	Source	IPBD	Links	Secreted	Organism	AfM	
IPBDs	Aprotinin	58	X-ray, NMR	3 SS	yes	Bos taurus	trypsin	5-55, 14-38	30-51 (1:6, 2:4, Preferred

3:5) Crambin 46 X-ray, NMR 3 SS yes rape seed ?, Mab CMTI-III 26 NMR 3 SS yes cucumber trypsin ST-I.sub.A 13 NMR 3 SS yes E. coli MAbs & guanylate cyclase Third domain, 56 X-ray, NMR 3 SS yes Coturnix trypsin ovomucoid coturnix japonica Ribonuclease A 124 X-ray, NMR yes Bos taurus RNA, DNA Ribonuclease 104 X-ray, NMR? yes A. oruzae RNA, DNA Lysozyme 129 X-ray, NMR? 4 SS yes Gallus gallus NAG-NAM-NAG Azurin 128 X-ray Cu:CYS, P. aerugenosa Mab HIS.sup.2, MET Characteristics of Known IPBDs .alpha.-Conotoxins 13-15 NMR 2 SS yes Conus snails Receptor .mu.-Conotoxins 20-25 NMR 3 SS yes Conus snails Receptor .OMEGA.-Conotoxins 25-30 -- 3 SS yes Conus snails Receptor King-kong 25-30 -- 3 SS yes Conus snails Mabs peptides Nuclease 141 X-ray none yes S aurius RNA, DNA (staphylococcal) Charybdotoxin 37 NMR 3 SS Yes Leiurus Ca.sup.+2 (scorpion toxin) 7-28, 13-33 17-35 guinguestriatus dependent (1:4, 2:5, 3:6) hebraeus K.sup.+ channel Apamin 12 NMR 2 SS yes Bees Mabs, (bee venom) (1:3, 2:4) Receptor (?) Other suitable IPBDs Ferredoxin Secretory trypsin inhibitor Soybean trypsin inhibitor SLPI (Secretory Leukocyte Protease Inhibitor) (THOM86) and SPAI (ARAK90) Cystatin and homologues (MACH89, STUB90) Eglin (MCPH85) Barley inhibitor (CLOR87a, CLOR87b, SVEN82)

CLAIMS:

7. The method of claim 3 wherein the initially chosen parental potential binding domain is selected from the group consisting of (a) binding domains of bovine pancreatic trypsin inhibitor, crambin, Cucurbita maxima trypsin inhibitor III, heat-stable enterotoxin of Excherichia coli, .alpha. Conotoxin GI, .mu.Conotoxin GIII, .omega. Conotoxin GIV, apamin, charybdotoxin, secretory leukocyte protease inhibitor, cystatin, eglin, barley protease inhibitor, ovomucoid, T4 lysozyme, hen egg white lysozyme, ribonuclease, azurin, tumor necrosis factor, and CD4, and (b) domains at least substantially homologous with any of the foregoing domains which have a melting point of at least 50.degree. C.

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L1: Entry 4 of 13 File: USPT Jan 5, 1999

DOCUMENT-IDENTIFIER: US 5856090 A
TITLE: DNA-methylase linking reaction

Brief Summary Paragraph Right (20):
In one particularly preferred alternative, each of the polypeptide determinant genes within the plurality of plasmid-polypeptide determinant conjugates can be derived from a single parent polypeptide determinant gene by random mutagenesis. The parent polypeptide determinant gene can code for any polypeptide, including, but not limited to, glutathione S-transferase, estrogen receptor, triose phosphate isomerase, thrombin, plasminogen, tissue plasminogen activator, streptokinase, human insulin, erythropoietin, thrombopoietin, a fibrinogen type III domain or a protein including a fibrinogen type III domain, a DNA binding domain or a protein including a DNA binding domain, a helix-turn-helix DNA binding domain or a protein including a helix-turn-helix DNA binding domain, interleukin, HIV reverse transcriptase, HIV protease, renin, elastase, subtilisin, .alpha.-lytic protease, hirudin, omatin, kistin, or eglin C. In one preferred alternative, the parent polypeptide determinant gene is glutathione S-transferase.

Detailed Description Paragraph Right (43):
The polypeptide determinant can be any single polypeptide chain expressible in a prokaryotic system, generally E. coli. There are no fixed length restrictions on the polypeptide determinant; it can be a short peptide or a long protein chain. It can be an intact protein or single subunit of a multi-subunit protein. Alternatively, it can be a structural or functional domain of a protein, or a fragment of a protein or peptide produced by proteolytic cleavage, either chemical or enzymatic. The polypeptide determinant can also be a synthetic or naturally occurring peptide. Typical polypeptide determinants include, but are not necessarily limited to, glutathione S-transferase, estrogen receptor, triose phosphate isomerase, thrombin, plasminogen, tissue plasminogen activator, streptokinase, human insulin, erythropoietin, thrombopoietin, a fibrinogen type III domain or a protein including a fibrinogen type III domain, a DNA binding domain or a protein including a binding domain, a helix-turn-helix DNA binding domain or a protein including a helix-turn-helix DNA binding domain, interleukin, interferon, HIV reverse transcriptase, HIV protease, renin, elastase, subtilisin, .alpha.-lytic protease, hirudin, omatin, kistin, and eglin C.

CLAIMS:

35. The library of claim 34 wherein the parent polypeptide determinant gene encodes a protein selected from the group consisting of glutathione S-transferase, estrogen receptor, triose phosphate isomerase, thrombin, plasminogen, tissue plasminogen activator, streptokinase, human insulin, erythropoietin, thrombopoietin, a fibrinogen type III domain or a protein including a fibrinogen type III domain, a DNA binding domain or a protein including a DNA binding domain, a helix-turn-helix DNA binding domain or a protein including a helix-turn-helix DNA binding domain, interleukin, HIV reverse transcriptase, HIV protease, renin, elastase, subtilisin, .alpha.-lytic protease, hirudin, omatin, kistin, and eglin C.

43. The method of claim 36 wherein the polypeptide determinant gene encodes a protein selected from the group consisting of glutathione S-transferase, estrogen receptor, triose phosphate isomerase, thrombin, plasminogen, tissue plasminogen activator, streptokinase, human insulin, erythropoietin, thrombopoietin, a fibrinogen type III domain or a protein including a fibrinogen type III domain, a DNA binding domain or a

protein including a DNA binding domain, a helix-turn-helix DNA binding domain or a protein including a helix-turn-helix DNA binding domain, interleukin, HIV reverse transcriptase, HIV protease, renin, elastase, subtilisin, .alpha.-lytic protease, hirudin, omatin, kistin, and eglin C.

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L1: Entry 2 of 13 File: USPT Aug 31, 1999

DOCUMENT-IDENTIFIER: US 5945297 A
TITLE: Process for screening candidate agents that modulate the hemostatic system

Brief Summary Paragraph Right (3):
The clotting cascade of the hemostatic process is initiated when trauma, surgery or disease disrupt the vascular endothelial lining and blood is exposed to subendothelial connective tissue. The injured vascular endothelial cell releases substances that initiate the clotting cascade, a process involving the activation of a series of compounds that ultimately results in the conversion of prothrombin to thrombin. Thrombin is a key enzyme in the coagulation event, catalyzing the activation of platelets and the cleavage of fibrinogen.

Brief Summary Paragraph Right (5):
Platelets are small disc-shaped cell fragments formed by the breakdown of megakaryocytes, and are very susceptible to changes in their environment such as pH and temperature. In normal blood banking procedures, donated whole blood is stored at a temperature of approximately 4.degree. C. Upon refrigeration, the platelet irreversibly changes its structural conformation from a disc shape to a sphere. When the platelet is not in the normal disc shape, it is incapable of aggregation and thus not physiologically relevant. In addition to the activation of platelets, thrombin also acts on fibrinogen, the key structural protein in blood clot formation and the substrate for thrombin proteolytic activity. Thrombin catalyzes the release of small peptides, fibrinopeptides A and B, from the chains of fibrinogen. The removal of the fibrinopeptides from the fibrinogen substrate results in the formation of fibrin monomers which polymerize into fibers and provide the structural matrix of the clot. The clot formed by fibrin is removed or degraded by the process of fibrinolysis. Fibrinolysis is initiated by the release of either tissue plasminogen activator or prourokinase from endothelial cells. These agents convert plasminogen into the active proteolytic enzyme, plasmin which catalyzes the fibrin substrate into soluble degradation products. In addition, plasmin is enzymatically active against fibrinogen, and degrades fibrinogen into soluble products.

Brief Summary Paragraph Right (11):
It is yet another object of this invention to provide an assay for the screening of candidate drugs that either inactivate or stimulate tissue plasminogen activator (tPA).

Drawing Description Paragraph Right (2):
FIG. 2 is a graph showing the effect of PAI-1 (plasminogen activator inhibitor) concentration to inhibit a single concentration of tPA within 90 min. The Y axis represents the total [.sup.125 I]-labeled fibrin remaining within the clot. The X axis represents the concentration of PAI-1. As the concentration of PAI-1 increases, total clot lysis decreases.

Detailed Description Paragraph Right (6):
By "a component of the hemostatic system" is meant any molecule or functional equivalent thereof that either directly or indirectly has a role in the hemostatic system, such as nucleosides, lipids, peptides or proteins. Examples include, but are not limited to, serine proteases like tissue-type plasminogen activator (tPA), urokinase or urinary-type plasminogen activator (uPA), plasmin, kallikrein, thrombin, Factors VIIa, IXa, Xa, XIa and XIIa, serine protease inhibitors, such as plasminogen activator inhibitor-1 (PAI-1), PAI-2 and PAI-3, .alpha.-2-antiplasmin, antithrombin III, and heparin cofactor I.

Detailed Description Paragraph Right (20):
Thus, for example, the screening of candidate agents that inhibit PAI-1 activity, may require the addition of thrombin, tPA and PAI-1. Alternatively, screening for candidates that promote clot formation may require the addition of tissue factor, Factor XIIa, XIa, IXa, VIII, Xa or thrombin. The effect of the candidate agent on the component of interest in the hemostatic system may be evaluated, as noted above, by conventional methodology. For example, the ability of a candidate agent to inhibit PAI-1 activity may be assessed directly by monitoring the activity of PAI-1 enzyme itself using a calorimetric assay such as the S-2288 assay sold by Chromogenix, Moindal, Sweden. Alternatively, the ability of a candidate agent to inhibit PAI-1 may be assessed indirectly by examining clot lysis. For example, a trace amount of labeled fibrinogen may be added to the assay prior to addition of the candidate agent. The labeled fibrinogen will be incorporated into the clot during clot formation. If the candidate agent inhibits PAI-1 activity, there should be a decrease in label incorporated into the clot during clot lysis and an increase in label in the buffer solution. The fibrinogen may be labeled by any conventional methodology. Examples of suitable labels include radioactive labels such as .sup.125 I, .sup.35 S, .sup.14 C, .sup.32 P, .sup.33 P, .sup.3 H, and the like. Fluorescent labels may be used as well. As will be appreciated by those skilled in the art, suitable fluorescent labels include, but are not limited to, fluorescein and fluorescein derivatives such as carboxyfluorescein, fluorescein acrylamide, fluorescein isothiocyanate, coumarin, seminaphthorhodafluorescein, seminaphthofluorescein, naphthofluorescein, hydroxypyrene trisulfonic acid and dichlorofluorescein.

Detailed Description Paragraph Right (23):
By way of example, particularly useful candidate agents to identify are those capable of modulating serine protease activity. For example, the chymotrypsin superfamily of serine proteases includes several proteases involved in regulating the hemostatic system, e.g., tissue-type plasminogen activator (tPA), urokinase or urinary-type plasminogen activator (uPA), plasmin, and clot cascade proteases including kallikrein, thrombin, and Factors VIIa, IXa, Xa, XIa and XIIa. The specificity of the bonds cleaved by these proteases ranges from the very broad, as in plasmin, to the extremely specific, as, for example, in the case of the clotting cascade enzymes. The activity of these proteases is regulated at several levels. Many of these proteases are produced in inactive, or zymogen, forms. The zymogen forms can be activated, for example, by cleavage of inhibitory domains within the molecules, thereby permitting the proteolytic activity of the enzyme to be revealed (for example, the cleavage of plasminogen to plasmin can be catalyzed by either uPA or tPA). Another means by which the activity of serine proteases is regulated involves polypeptides that directly inhibit proteolytic activity. Inhibition is accomplished by employing a protein structure which fits within the active site of a particular serine protease. Therefore, examples of serine protease inhibitors that can be screened by the instant method include, but are not limited to, the bovine pancreatic trypsin inhibitor ("BPTI") family (e.g., snake venom inhibitor, inter-alpha inhibitor, and the A4 amyloid precursor "A4695"), the Kazal family (e.g., pancreatic secretory inhibitor, ovomucoid, and seminal plasma acrosin inhibitor), the Streptomyces subtilisin family (e.g., from S. albogriseolus, and plasminostreptin), the serpin family (e.g., plasminogen activator inhibitor-1 (PAI-1), PAI-2, PAI-3, C1 esterase inhibitor, .alpha.-2-antiplasmin, contrapsin, .alpha.-1-antitrypsin, antithrombin III, protease nexin I, .alpha.-1-antichymotrypsin, protein C inhibitor, heparin cofactor II and growth hormone regulated protein), the soybean trypsin inhibitor family (e.g., soybean trypsin inhibitor), the potato inhibitor family (e.g., potato inhibitor, barley chymotrypsin inhibitor, and leech inhibitor eglin), and the Bowman-Birk inhibitor family (e.g., Lima bean inhibitor IV, garden bean inhibitor, and adzuki bean inhibitor II).

Detailed Description Paragraph Right (24):
Alternatively, because thrombolytic agents are increasingly used in the prevention and/or dissolution of formed thrombi such as in acute myocardial infarction or stroke, it would be useful to identify candidate agents that inhibit the ability of PAI-1 to inactivate tPA- and uPA-catalyzed conversion of plasminogen to plasmin. Potential candidate agents to be screened may be selected from diketopiperazine compounds or derivatives or analogs thereof (see, e.g., International Patent Publ. Nos. WO 95/21832, WO 95/32190, WO 95/21829 and UK Patent GB 2 284 420 B, Bryans et al. (1996)

J Antibiotics 49:1014, Charlton et al. (1996) Thrombosis and Haemostasis 75:808, Charlton (1997) Exp. Opin. Invest. Drugs 6:539, Charlton (1997) Drugs of the Future 29:45, and Charlton et al.(1997) Fibrinolysis & Proteolysis 11:51).

Detailed Description Paragraph Right (26):
Some patients who have very limited ability to form clots could also benefit from a candidate agent that has been screened using the process of the invention as a suitable drug for promoting the clotting cascade. Examples of diseases or disorders where such drugs would have therapeutic potential, include, but are not limited to afibrinogenemia, dysfibrinogenemia, hypoprothrombinemia, parahemophelia, hypoconvertinemia, hemophilia A, hemophilia B, Stuart-Prower factor deficiency, plasma thromboplastin antecedent deficiency, Hageman trait, thrombocytopenia, disorders of platelet function, von Willebrand's disease, hepatic dysfunction, circulating anticoagulants, inherited defects in natural coagulation inhibitors (such as antithrombin, protein C, or protein S), dysplasminogenemia, defective release or diminished venous content of plasminogen activator, excessive release of PAI, heparin cofactor II deficiency, homocystinuria, chronic congestive heart failure, metastatic tumor or malignancy, extensive trauma or major surgery, myeloproliferative disorders, or treatment with oral contraceptives or L-asparaginase.

Detailed Description Paragraph Right (33):
Compatibility matched QNS (quantity non-sufficient) whole blood and outdated single platelet packs were purchased from Peninsula Blood Bank (Burlingame, Calif.). Two-chain tPA was purchased from Biopool (Umea, Sweden). Fibrinogen (.sup.125 I) 7.38 MBq/mg, 110 .mu.Ci was purchased from Amersham (Arlington Heights, Ill.). Thrombin and low molecular weight heparin were purchased from Sigma (St. Louis, Mo.). Optiphase Supermix scintillation cocktail was purchased from Wallac (Turku, Finland). Anti-PAI-1 monoclonal Ab 3783 was from American Diagnostica, Inc. (Greenwich, Conn.). Anti-PAI-1 peptide was purchased from Peninsula Laboratories (Belmont, Calif.).

Detailed Description Paragraph Right (34):
Platelets (150 .mu.l) were added to 1.2 ml hypotonic phosphate buffer, containing 53 mM Na.sub.2 HPO.sub.4 and 12 mM KH.sub.2 PO.sub.4. To this solution 150 .mu.l whole blood (less than 23 days old), supplemented with .about.120 nCi [.sup.125 I]-labeled fibrinogen, was added followed by the addition of tPA (0.5-15 IU). The reaction was gently mixed in a 17.times.100 mm polypropylene tube in the presence of 1.25 units thrombin. After 5 min. or upon clot formation the tubes were gently shaken to dislodge the clots from the side of the tubes to hasten retraction. After 90 min. the reaction was terminated by the addition of 10 ml phosphate buffer. The diluted reaction volume was immediately poured over a 25 mm Whatman GF/C filter. The filters were washed with an additional 5 ml phosphate buffer, and then dried at 55.degree. C. for 30 min. and scintillation counted. Fibrinolysis was measured as the difference between total counts in the reaction and total counts remaining on the filters. The reactions were carried out a minimum of four times in duplicate. Results are illustrated in FIG. 1, a graph showing the concentration-dependent activity of tPA on fibrinolysis. The Y axis represents the total [.sup.125 I]-labeled fibrin remaining in the clot after 90 minutes, and the X axis represents the concentration of tPA. As the clot is lysed, less [.sup.125 I]-labeled fibrin is detected in the clot, and as the concentration of tPA increases, total clot lysis is increased as well.

Detailed Description Paragraph Right (36):
To demonstrate the use of the assay of the invention as a potential screen for inhibitors of thrombotic molecules such as PAI-1, the effect of a PAI-1 inhibitor, an anti-PAI-1 peptide, was evaluated for its capability to reduce PAI-1 inhibition of tPA. The anti-PAI-1 peptide is a fourteen amino acid peptide homologous to the .alpha. segment of the active loop of the PAI-1 molecule. This peptide is reported to inhibit the function of PAI-1 at 50 .mu.M in a dilute blood clot lysis assay using classical methods (see Eitzman et al. (1995) J. Clin. Invest. 95:2416-2420). In this assay the addition of buffer, platelets and [.sup.125 I]-labeled fibrinogen supplemented whole blood was added as described above. In each reaction tube 24 IU tPA was added followed by 1.2 nM PAI-1 15 min. preincubation +/-anti-PAI-1 peptide (0.5-50 .mu.M). Each reaction was gently mixed and 1.25 units thrombin was added. After 90 min. reaction time, samples were treated as described above. Results are illustrated in FIG. 5, a graph illustrating inhibition of PAI-1 by the anti-PAI-1 peptide. In the graph, the Y axis represents the total [.sup.125 I]-labeled fibrin retained within the clot after

90 min., and the X axis represents the effect of a single concentration of tPA and a single concentration of PAI-1 in the presence of increasing concentrations of the anti-PAI-1 peptide. At a concentration of 10 .mu.M, complete inhibition of PAI-1 was observed.

Detailed Description Paragraph Right (38):
Experiments were carried out to demonstrate the versatility of the assay of the invention to detect the effect of the small molecule XR-5118 on PAI-1. The small molecule XR-5118 is reported to be an inhibitor of PAI-1 and belongs to a class of compounds known as diketopiperazines. See, for example, Charlton et al. (1997), "XR5118, A Novel Modulator of Plasminogen Activator Inhibitor-1 (PAI-1), Increases Endogenous tPA Activity in the Rat," Fibrinolysis & Proteolysis 11(1):51-56. XR-5118 ((3Z,6Z)-6-benzylidene-3-(5-(2-dimethylaminoethylthio)-2-thienyl)methylene -2,5-piperazinedione, hydrochloride) was synthesized as described in PCT Publication No. WO 95/32190 (Bryans et al., "Pharmaceutical Diketopiperazine Compounds). The addition of buffer, platelets and radiolabled whole blood was as described above. In each reaction tube 12 IU tPA was added. Reaction tubes were treated with a single concentration of PAI-1 (0.2-10.0 nM) in the presence or absence of 30 .mu.M XR-5118. After gentle mixing each tube was treated with 1.25 units thrombin. Reaction mixtures were incubated for 90 min. and then processed as described above. Results are illustrated in FIG. 4, a graph illustrating inhibition of PAI-1 by the small molecule XR-5118. In the graph, the Y axis represents the total [.sup.125 I]-labeled fibrin remaining within the clot after 90 min., while the X axis represents the effect of a single concentration of tPA in the presence of increasing concentrations of PAI-1 in the presence or absence of XR-5118 (30 .mu.M). In the presence of XR-5118, PAI-1 activity was found to decrease approximately 35-45%.

Detailed Description Paragraph Right (39):
This experiment demonstrates the functionality of various bioactive molecules, such as antithrombin, in the assay of the invention, and also demonstrates the use of the assay as a potential screen for inhibitors of thrombin-stimulated clot formation, e.g., heparin. In this assay, the addition of buffer, platelets and [.sup.125 I]-labeled fibrinogen supplemental whole blood was added as described above. A reaction tube was then treated with a single concentration of heparin (0.05-3.0 U/ml), prior to the addition of thrombin (1.25 units). The reaction mixture was incubated for 90 min. and treated as described above. The experiment was carried out three times in duplicate. Results of this experiment showing the effect of low molecular weight heparin on thrombin-induced clot formation are set forth in the graph of FIG. 6. In FIG. 6, the Y axis represents the total [.sup.125 I]-labeled fibrin remaining within the clot after 90 min. The X axis represents the concentration of heparin used.

Other Reference Publication (3):
Charlton et al. (1996), "Evaluation of a Low Molecular Weight Modulator of Human Plasminogen Activator Inhibitor-1 Activity," Thrombosis and Haemostasis 75(5):808-815.

Other Reference Publication (4):
Charlton et al. (1997), "XR5118, A Novel Modulator of Plasminogen Activator Inhibitor-1 (PAI-1), Increases Endogenous tPA Activity in the Rat," Fibrinolysis & Proteolysis 11(1):51-56.

CLAIMS:

21. The method of claim 1, wherein the candidate agent is a potential inhibitor of plasminogen activator inhibitor (PAI-1).

22. The method of claim 1 further comprising, prior to step (c), adding to the screening medium a compound selected from the group consisting of thrombin, tissue plasminogen activator, fibrinogen, tissue factor, Factors XIIa, XIa, IXa, VIII and Xa.

23. The method of claim 1, further comprising the step of adding labeled fibrinogen to the screening medium prior to step (c).

24. The method of claim 23, wherein the fibrinogen is radioactively labeled.

25. The method of claim 24, wherein the fibrinogen is radioactively labeled with a label selected from the group consisting of .sup.125 I, .sup.35 S, .sup.14 C, .sup.32 P, .sup.33 P and .sup.3 H.

26. The method of claim 23, wherein the fibrinogen is labeled with a fluorescent label.

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L1: Entry 1 of 13 File: USPT May 9, 2000

DOCUMENT-IDENTIFIER: US 6060451 A
TITLE: Thrombin inhibitors based on the amino acid sequence of hirudin

Brief Summary Paragraph Right (6):
Thrombin is an important serine proteinase component of the blood coagulation cascade. Besides initiating blood clotting by cleaving fibrinogen, thrombin activates other hemocoagulant factors including factors V, VIII and XIII and the anticoagulant enzyme protein C. Thrombin is also a potent platelet activator that impairs thrombolysis mediated by tissue plasminogen activator in vivo. Thus, thrombin's positive feedback regulation serves to amplify hemostatic events but causes life-threatening thrombi in response to aberrations with vascular and cerebrovascular arteries.

Brief Summary Paragraph Right (12):
It has been found that native desulfo hirudin.sup.45-65 inhibits fibrinogen clotting by both bovine and human .alpha.-thrombin in a dose dependent manner. The IC.sub.50 value of 940.+-.200 nM for bovine .alpha.-thrombin is in good agreement with the reported value of plasma fibrin clot formation by the same fragment and three times lower than hirudin.sup.55-65, which had been assigned as the minimum core required for anticoagulant activity. It has also been demonstrated that the same peptides were consistently more potent against human .alpha.-thrombin than bovine .alpha.-thrombin.

Brief Summary Paragraph Right (17):
In their conclusion, the authors mention that the C-terminal region of hirudin may bind to a region of fibrinogen binding on thrombin that is not the region proposed so far in the literature.

Brief Summary Paragraph Right (21):
Finally, Maraganore et al., in "Anticoagulant activity of synthetic hirudin peptides", (1989), The Journal of Biological Chemistry, Vol. 264, No. 15, pages 8692-8698, Dennis et al. in "Use of fragments of hirudin to investigate thrombin-hirudin interaction", (1990), Eur. J. Biochem. 188, pages 61-66 and Chang et al. in "The structural elements of hirudin which bind to the fibrinogen recognition site of thrombin are exclusively located within its acidic C-terminal tail", (1990), Febs., Vol. 261, No. 2, pages 287-290, describe the synthesis and anticoagulant properties of a number of peptides whose sequences are based on the sequence of various fragments of native hirudin.

Brief Summary Paragraph Right (24):
The design of low molecular weight and specific inhibitors of thrombin that utilize accessory binding loci remote from or in conjunction with the catalytic center, similar to the way fibrinogen or hirudin binds to thrombin, constitutes a challenge in protein chemistry. Conceivably, such a multifunctional inhibitor integrates two or more recognitive elements, separated by a suitable spacer, that favor multiple simultaneous interactions and which could manifest enhanced potency and specificity. Incorporation of "foreign" chemical elements embodied in a structure of low molecular weight could confer resistance against proteolysis and favourable bioavailability. Also, because they are smaller than hirudin, these compounds are less likely to stimulate an undesirable immune response in patients treated with them.

Detailed Description Paragraph Right (79):
The peptides of the present invention may be administered alone or in combination with other pharmaceuticals. For example, the peptides may be administered in combination with tissue plasminogen activator to prevent reocclusion of coronary arteries. Alternatively, the peptides of the present invention could be administered with

heparin or low molecular weight heparin, a combination which could advantageously lower the dosage of heparin or low molecular weight heparin.

Detailed Description Paragraph Right (144):
Inhibition of fibrinogen clot formation was measured spectrophotometrically at 405 nm on a Varian DMS 90 at 37.degree. C. 300 .mu.L of 0.1% fibrinogen (Sigma) in 0.1M Tris-HCl, pH 7.8, containing 0.1M NaCl, 0.1% PEG 600 and variable concentrations of inhibitor in the same buffer were mixed in polystyrene cuvettes and the reaction was initiated by the addition of the enzyme (human or bovine .alpha.-thrombin 0.4 nM) in a total volume of 1 mL. The time from mixing to inflection due to clot formation was recorded for various inhibitor concentrations and IC50 values were calculated by log probit analysis. The concentrations of the inhibitors in the assays was based on the peptide content.

Detailed Description Paragraph Right (150):
The various experiments have also demonstrated that while D-Phe-Pro-Arg-Pro-OH and N.alpha.-acetyl desulfo hirudin.sup.55-65 independently inhibited fibrin clot formation by bovine .alpha.-thrombin with IC.sub.50 values of 250 .mu.M and 3.5 .mu.M respectively, their incorporation into a single molecule separated by a spacer corresponding to hirudin residues 49-54 afforded an inhibitor with an IC.sub.50 =70.+-.20 nM (bovine .alpha.-thrombin) and 4.+-.0.8 nM (human .alpha.-thrombin). The effect of combining separate IC.sub.50 doses of hirudin.sup.45-65 and D-Phe-Pro-Arg-Pro-OH resulted only in the doubling of the fibrinogen clotting time, while the contribution of the spacer was negligible. The synergistic effect observed for P53 in the clotting assay was corroborated by results of the fluorogenic assay where this analog emerged as a pure competitive inhibitor compared to P51 with Ki values

Detailed Description Paragraph Left (9):
Fibrinogen Clotting Assay

Detailed Description Paragraph Table (3):
TABLE III
Effect on antithrombin activity in vitro and on antithrombotic activity in vivo in a model of ferric chloride injury-induced thrombosis in carotid artery in rats. IC50 IC50 plasma Dose to double thrombin-induced P number Ki IC50 clotting time patency time platelet aggregation (BCH number) (pM) dTT (nM)* (nM)** (mg/kg) (nM)

P79 (SEQ ID NO: 1) (1709) 500 1.9 9 1 2 (1710) (SEQ ID NO: 6) 1500 10.5 52 1-2 6 P109 (SEQ ID NO: 4) (1711) 2250 ND 38 ND ND P290 (SEQ ID NO: 8) (1719) 900 ND 11 ND ND P311 (SEQ ID NO: 9) (1721) 160 1.35 3 ND ND P314 (SEQ ID NO: 10) (1726) 20 1.3 1.4 1 4.5 P582 0.053 ND ND ND ND P596 (SEQ ID NO: 13) 0.046 ND ND ND ND 0.051.sup.2 P603 (SEQ ID NO: 15) 0.067 ND ND ND ND P604 (SEQ ID NO: 16) 0.050 ND ND ND ND P605 (SEQ ID NO: 17) 0.068 ND ND ND ND P606 (SEQ ID NO: 18) 0.040 ND ND ND ND 0.073.sup.2 (2408) (SEQ ID NO: 26) 75 1 1.8 0.5 11 (2414) (SEQ ID NO: 27) 1000 6.23 10.5 ND ND (2423) (SEQ ID NO: 28) 450 1.15 10 1 135 (2739) (SEQ ID NO: 29) 4000 14.5 78 1 20 (2757) (SEQ ID NO: 30) 11000 25.5 190 >2 50 (2758) (SEQ ID NO: 31) 950 4.9 30 4 10 (2763) (SEQ ID NO: 32) 100 1.3 3.1 1 0.7 (2767) (SEQ ID NO: 33) 40000 165 700 >2 ND r-Hirudin 0.4 ND ND 1 ND

The dose of heparin needed to cause a doubling in patency time is 200 U/kg. ND denotes not determined. Values are means for 3-5 observations. Patency time in control (salinetreated) rats is 19 .+-. 1 min (n = 11). *Concentration of compound required to double thrombin time in buffer containing fibrinogen. **Concentration of compound required to inhibit human plasma clotting tim by 50%. 1 first test; 2 second test

Other Reference Publication (9):
Chang et al., "The Structural Elements of Hirudin which Bind to the Fibrinogen Recognition Site of Thrombin Are Exclusively Located within its Acidic C-Terminal Tail," FEBS Letters, vol. 261, No. 2, pp. 287-290 (1990).

Other Reference Publication (15):
Seemuller et al., "Proteinase Inhibitors of the Leech Hirudo medicinalis (Hirudins, Bdellins, Eglins)." No year given.

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L2: Entry 1 of 2 File: USPT Jan 5, 1999

DOCUMENT-IDENTIFIER: US 5856090 A
TITLE: DNA-methylase linking reaction

Brief Summary Paragraph Right (20):
In one particularly preferred alternative, each of the polypeptide determinant genes within the plurality of plasmid-polypeptide determinant conjugates can be derived from a single parent polypeptide determinant gene by random mutagenesis. The parent polypeptide determinant gene can code for any polypeptide, including, but not limited to, glutathione S-transferase, estrogen receptor, triose phosphate isomerase, thrombin, plasminogen, tissue plasminogen activator, streptokinase, human insulin, erythropoietin, thrombopoietin, a fibrinogen type III domain or a protein including a fibrinogen type III domain, a DNA binding domain or a protein including a DNA binding domain, a helix-turn-helix DNA binding domain or a protein including a helix-turn-helix DNA binding domain, interleukin, HIV reverse transcriptase, HIV protease, renin, elastase, subtilisin, .alpha.-lytic protease, hirudin, omatin, kistin, or eglin C. In one preferred alternative, the parent polypeptide determinant gene is glutathione S-transferase.

Detailed Description Paragraph Right (43):
The polypeptide determinant can be any single polypeptide chain expressible in a prokaryotic system, generally E. coli. There are no fixed length restrictions on the polypeptide determinant; it can be a short peptide or a long protein chain. It can be an intact protein or single subunit of a multi-subunit protein. Alternatively, it can be a structural or functional domain of a protein, or a fragment of a protein or peptide produced by proteolytic cleavage, either chemical or enzymatic. The polypeptide determinant can also be a synthetic or naturally occurring peptide. Typical polypeptide determinants include, but are not necessarily limited to, glutathione S-transferase, estrogen receptor, triose phosphate isomerase, thrombin, plasminogen, tissue plasminogen activator, streptokinase, human insulin, erythropoietin, thrombopoietin, a fibrinogen type III domain or a protein including a fibrinogen type III domain, a DNA binding domain or a protein including a binding domain, a helix-turn-helix DNA binding domain or a protein including a helix-turn-helix DNA binding domain, interleukin, interferon, HIV reverse transcriptase, HIV protease, renin, elastase, subtilisin, .alpha.-lytic protease, hirudin, omatin, kistin, and eglin C.

CLAIMS:

35. The library of claim 34 wherein the parent polypeptide determinant gene encodes a protein selected from the group consisting of glutathione S-transferase, estrogen receptor, triose phosphate isomerase, thrombin, plasminogen, tissue plasminogen activator, streptokinase, human insulin, erythropoietin, thrombopoietin, a fibrinogen type III domain or a protein including a fibrinogen type III domain, a DNA binding domain or a protein including a DNA binding domain, a helix-turn-helix DNA binding domain or a protein including a helix-turn-helix DNA binding domain, interleukin, HIV reverse transcriptase, HIV protease, renin, elastase, subtilisin, .alpha.-lytic protease, hirudin, omatin, kistin, and eglin C.

43. The method of claim 36 wherein the polypeptide determinant gene encodes a protein selected from the group consisting of glutathione S-transferase, estrogen receptor, triose phosphate isomerase, thrombin, plasminogen, tissue plasminogen activator, streptokinase, human insulin, erythropoietin, thrombopoietin, a fibrinogen type III domain or a protein including a fibrinogen type III domain, a DNA binding domain or a

protein including a DNA binding domain, a helix-turn-helix DNA binding domain or a protein including a helix-turn-helix DNA binding domain, interleukin, HIV reverse transcriptase, HIV protease, renin, elastase, subtilisin, .alpha.-lytic protease, hirudin, omatin, kistin, and eglin C.

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L3: Entry 5 of 10 File: USPT Sep 26, 2000

DOCUMENT-IDENTIFIER: US 6124107 A
TITLE: Assay for marker of human polymorphonuclear leukocyte elastase activity

Brief Summary Paragraph Right (1):
Human polymorphonuclear leukocyte elastase (PMNE) cleaves human fibrinogen at multiple sites. Cleavage of the A.alpha. chain at A.alpha.(Val.sup.360 -Ser.sup.361) generates a stable product as indicated by its presence in biological fluids. A radioimmunoassay (RIA) based on the A.alpha.(Val.sup.360) epitope of this cleavage site has been developed which allows the evaluation of the potency of elastase inhibitors to inhibit formation of cleavage products containing this neoepitope in a variety of in vitro cell biological situations. The RIA detects an endogenous A.alpha.(Val.sup.360) in normal human plasma and at elevated concentrations in cystic fibrosis plasma and in rheumatoid arthritis synovial fluid samples.

Brief Summary Paragraph Right (13):
Human fibrinogen is a hetero-dimeric glycoprotein consisting of 3 non-identical chains, A.alpha., B.beta. and .gamma.. PMNE cleaves human fibrinogen at multiple sites. Primary sites of cleavage include A.alpha.(Val.sup.21 -Glu.sup.22), A.alpha.(Val.sup.360 -Ser.sup.361), A.alpha.(Val.sup.450 -Ile.sup.451), A.alpha.(Val.sup.464 -Thr.sup.465), A.alpha.(Met.sup.476 -Asp.sup.477), A.alpha.(Thr.sup.568 -Ser.sup.569), .gamma.(Thr.sup.305 -Ser.sup.306), .gamma.(Val.sup.347 -Tyr.sup.348) and .gamma.(Ala.sup.357 -Ser.sup.358). We have developed two antipeptide antibodies, one of which specifically measures PMNE hydrolysis of fibrinogen at the A.alpha.(Val.sup.21 -Glu.sup.22) position to release a 21 residue N-terminal peptide, and a second which measures cleavage at A.alpha.(Val.sup.360 -Ser.sup.361), to release a 250 residue C-terminal fragment (FIG. 1). The A.alpha.(Val.sup.360) carboxyl terminal fragment remains associated with the .beta. and .gamma. chains of fibrinogen due to the disulfide network of the protein. Neither of the 2 specific antisera recognize intact fibrinogen. Both of these RIA allow the evaluation of the potency of PMNE inhibitors, such as elastase inhibitors, to inhibit fibrinopeptide neoepitope generation in whole blood stimulated with the calcium ionophore A23187. However, a major disadvantage of the A.alpha.(Val.sup.21) assay is the rapid in vivo clearance and metabolism of the peptide neoepitope A.alpha.(Val.sup.21) (t.sub.1/2 of 30 sec in both the dog and rhesus monkey). In an extensive series of experiments we were unable to detect the A.alpha.(Val.sup.21) neoepitope in normal human plasma or in plasma samples from PiZZ individuals, nor in plasma from from patients with cystic fibrosis, emphysema or chronic bronchitis.

Detailed Description Paragraph Right (3):
Human fibrinogen is a hetero-dimeric glycoprotein consisting of 3 non-identical chains, A.alpha., B.beta. and .gamma.. PMNE cleaves human fibrinogen at multiple sites. Primary sites of cleavage include A.alpha.(Val.sup.21 -Glu.sup.22), A.alpha.(Val.sup.360 -Ser.sup.361), A.alpha.(Val.sup.450 -Ile.sup.451), A.alpha.(Val.sup.464 -Thr.sup.465), A.alpha.(Met.sup.476 -Asp.sup.477), A.alpha.(Thr.sup.568 -Ser.sup.569), .gamma.(Thr.sup.305 -Ser.sup.306), .gamma.(Val.sup.347 -Tyr.sup.348) and .gamma.(Ala.sup.357 -Ser.sup.358). We have developed two antipeptide antibodies, one of which specifically measures PMNE hydrolysis of fibrinogen at the A.alpha.(Val.sup.21 -Glu.sup.22) position to release a 21 residue N-terminal peptide, and a second which measures cleavage at A.alpha.(Val.sup.360 -Ser.sup.361), to release a 250 residue C-terminal fragment (FIG. 1). The A.alpha.(Val.sup.360) carboxyl terminal fragment remains associated with the .beta. and .gamma. chains of fibrinogen due to the disulfide network of the protein. Neither of the 2 specific antisera recognize intact fibrinogen. Both of these RIAs

allow the evaluation of the potency of PMNE inhibitors, such as elastase inhibitors, to inhibit fibrinopeptide neoepitope generation in whole blood stimulated with the calcium ionophore A23187. However, a major disadvantage of the A.alpha.(Val.sup.21) assay is the rapid in vivo clearance and metabolism of the peptide neoepitope A.alpha.(Val.sup.21) (t.sub.1/2 of 30 sec in both the dog and rhesus monkey). In an extensive series of experiments we have been unable to detect the A.alpha.(Val.sup.21) neoepitope in normal human plasma or in plasma samples from PiZZ individuals, nor in plasma from from patients with cystic fibrosis, emphysema or chronic bronchitis.

Detailed Description Paragraph Right (37):
The above defined assay technology is used to monitor the activity of human elastase inhibitors of human leukocyte elastase activity in human and primate blood. Generally an elastase inhibitor is combined with whole blood or given to primates or humans and the effect of leukocyte elastase on fibrinogen is determined. Replicate aliquots of freshly-drawn heparinized whole human blood are prepared with concentrations of elastase inhibitor ranging up to about 300 .mu.g/ml. Following a brief pre-incubation with the incubator, a membrane perturbator, such as calcium ionophore A23187, is added at a concentration of between about 75 .mu.M and about 300 .mu.M. Non-membrane perturbator controls containing blood and perturbator-only controls are included to measure the extent of uninhibited peptide generation. All assay samples are incubated at about 37.degree. C. for about 25 minutes. The plasma is then prepared and assayed fibrinogen cleavage products as described above. Elastase inhibitors are capable of inhibiting the generation of fibrinogen cleavage products and the levels of inhibition are easily detected using this novel assay system.

Detailed Description Paragraph Right (38):
In vivo inhibition of fibrinogen cleavage products following treatment of primates with an elastase inhibitor is evaluated. Blood or fluid samples are collected both before and after treatment with either an elastase inhibitor or saline. Each heparinized blood sample is divided into about 4 aliquots (about 1 ml) and processed as described above. Treatment of an animal with an elastase inhibitor causes a marked reduction in the amount of elastase cleavage product produced.

Detailed Description Paragraph Right (39):
The ability of the novel assay to determine the presence of the unique fibrinogen cleavage products and to determine the relative amounts of these products is evaluated with blood from individuals genetically deficient in alpha 1-proteinase inhibitor (.alpha.1Pi), a normal serum elastase inhibitor. Individuals deficient in .alpha.1Pi, exhibit the PiZZ phenotype, and produce less than normal levels of circulating .alpha.1Pi which is a natural inhibitor of leukocyte elastase, Janoff, Am. Rev. Respir. Dis. 132: 417-433 (1985). Consequently individuals exhibiting the PIZZ phenotype would not have the capacity to inhibit elastase activity and they should have increased fibrinogen cleavage products when assayed by the above procedure. When heparinized blood is collected from individuals who possess the PiZZ phenotype and processed as described above and levels of specific cleavage peptide antigen are measured, they are higher than normal volunteers.

Detailed Description Paragraph Right (74):
The effect of the elastase inhibitor 3-Acetoxymethyl-1.alpha.-methoxy-6-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene -2-(2-(S)carboxy-pyrrolidinecarboxamide)5,5-dioxide (Compound 1) on the calcium ionophore A23187-induced fibrinogen cleavage peptide production was evaluated. Replicate 2 ml aliquots of freshly-drawn heparinized whole human blood were prepared with concentrations of Compound 1 ranging up to 100 .mu.l/ml. Following a brief pre-incubation of 5 minutes at 37.degree. C., calcium ionophore A23187 was added to a final concentration of 150 .mu.M. Non-ionophore containing blood and ionophore-only (no inhibitor) controls were included to measure the extent of uninhibited peptide generation. All aliquots were incubated at 37.degree. C. for 25 minutes, the plasma collected, processed and assayed for fibrinogen cleavage peptide as described above. The results are shown in the following table.

Detailed Description Paragraph Right (78):
The blood samples drawn from the treated chimpanzee after infusion of the elastase inhibitor produced markedly lower levels of the fibrinogen peptide in response to calcium ionophore A23187. Fibrinogen cleavage peptide was not detected in the

non-ionophore treated blood samples from either animal. Over the course of 30 to 40 minutes, the amount of ionophore-stimulated peptide production in freshly-drawn samples gradually returned to the pretreatment level. No consistent change over time was observed in the untreated animal.

Detailed Description Paragraph Center (26):
Effect of An Elastase Inhibitor on Calcium Ionophore A23187-Stimulated Production of Fibrinogen Cleavage Peptide In The Blood of Normal Humans

Detailed Description Paragraph Center (27):
Effect of An Elastase Inhibitor on Calcium Ionophore A23187-Stimulated Production of Fibrinogen Cleavage Peptide In Primate Blood